

Showman, Chemical and Microbial Ecology of 'Awa, *Piper methysticum* (G. Forst)

CHEMICAL AND MICROBIAL ECOLOGY
OF 'AWA, *PIPER METHYSTICUM* (G. FORST)

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By

Angelique F. Showman

Thesis Committee:

Jon-Paul Bingham, Chairperson

David Christopher

Pratibha Nerurkar

Helen Turner

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LIST OF ABBREVIATIONS

| | |
|------------------|---|
| Ω | ohm |
| 16S V4 rDNA | bacterial 16S V4 hypervariable region ribosomal DNA |
| ACE | Abundance-based Coverage Estimator |
| ANOVA | analysis of variance |
| AUC | area under the curve |
| BAPTA | 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (calcium specific chelator) |
| CB1 | cannabinoid receptor 1 |
| cm | centimeters |
| CNS | central nervous system |
| CRAC, I_{CRAC} | calcium release activate channel |
| CRT | control |
| Cy5 | cyanine dye |
| DCA | detrended correspondence analysis |
| DNA | deoxyribonucleic acid |
| DPSS | diode pump solid state |
| EGTA | ethylene glycol tetraacetic acid |
| FITC | fluorescein isothiocyanate |
| Fluo-4 | fluorescent calcium-sensitive dye, systematic name: 2,2'-((2-(2-(2-(bis(carboxymethyl)amino)-5-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)phenoxy)ethoxy)-4-methylphenyl)azanediyl)diacetic acid |
| GABA | gamma amino butyric acid |
| GLP | good laboratory practices |
| GLUT | glucose transporter |
| GPCR | G-protein-coupled receptors |
| GPS | global positioning system |
| HC-AN | hierarchical clustering - average-neighbor |
| HEPES | n-2-hydroxyethylpiperazine-n'-2-ethanesulphonic acid |
| HPLC | high performance liquid chromatography |
| HSD | honest significant difference |

| | |
|-----------------|--|
| Hz, kHz | hertz, kilohertz |
| Ins (1,4,5) P3 | inositol (1,4,5) trisphosphate |
| iTOL | interactive Tree of Life |
| min | minute |
| mL | millilitres |
| mOsm | milliosmolarity |
| mRNA | messenger RNA |
| mV | millivolt |
| mW | milliwatt |
| nm | nanometers |
| nM, μ M, mM | nanomolar, micromolar, millimolar |
| OTU | <u>operational</u> taxonomic unit |
| pA/pF | picoamperes per picofarad |
| PCoA | Principal Coordinate Analysis |
| PCR | polymerase chain reaction |
| QC/QA | quality control/quality assurance |
| RBL2H3 | rat basophilic leukaemia subtype 2H3 |
| RFU | relative fluorescence units |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| SD | standard deviation |
| SERCA | sarcoplasmic endoplasmic reticulum calcium ATPase |
| THC | tetrahydrocannabinol |
| TPK | traditional pharmacological knowledge |
| TPMPA | tetrahydropyridin-4-yl)methylphosphinic acid |
| TRP | transient receptor potential cation channel |
| TRPA1 | transient receptor potential cation channel, member A1 |
| TRPM2 | transient receptor potential cation channel, member M2 |
| TRPV1 | transient receptor potential cation channel, member V1 |
| TxRed | Texas red dye |
| μ m | micrometer |

ABSTRACT

Kava or 'Awa, *Piper methysticum* (G. Forst), is traditionally a social, medical and ceremonial drink used in Hawai'i, across the Pacific, and more recently, as a nutraceutical primarily for the treatment of anxiety. This thesis documents two distinct yet related projects on kava biology:

(1) Identification of physiological effects of kava exposure in cells of the immune system.

Rationale: The efficacy and possible toxicity of kava may reflect additional biological targets in addition to GABA receptors in the CNS. **Hypothesis:** Immune system cells and their calcium signaling channels (e.g. the TRP family that have been shown to react to plant secondary metabolites from diverse sources) may be novel targets for kava constituents. If validated, this hypothesis would illuminate new understandings of the therapeutic potential of kava, as well as the indigenous medicine indications of the drink. **Results:** Using whole cell patch clamping techniques, bulk cell calcium and single cell calcium assays and on the RBL2H3 cell model, we demonstrated that kava, as well as non-kava lactone components separated via reversed-phase HPLC, were active on these non-CNS cellular targets. Specifically, whole cell patch clamping revealed TRPV1-like, TRPM2-like and I_{CRAC} -like conductances. Bulk calcium assays showed at minimum that some non-kava lactone fractions initiated a minor release of both internal and external calcium stores, which was confirmed by single cell calcium assays indicating a significant difference ($p < 0.01$) was observed. We further characterized calcium responses demonstrated by non-fractionated kava extractions via calcium-stored depletion using thapsigargin, a known I_{CRAC} inductor, and calcium add-back experiments and learned that kava is able to enhance calcium influx responses above those initiated via I_{CRAC} depletion indicating that non- I_{CRAC} channels are being recruited and are not-overlapping. **Conclusions:** These results suggest that a diverse secondary metabolome in kava is likely to affect the physiological and pathophysiological response in cells via compounds other than kavalactones and via targets other than GABA-R. Future studies would focus upon identification by fractionation of the individual secondary metabolites that are regulating human immune cell calcium signaling pathways.

(2) Pilot evaluation of the kava plant associated microbiome. Rationale: Production of these secondary metabolites that regulate human physiology are heavily affected by changes in the physical and biological environment of the whole plant, including the plant's associated microbial community. The first step towards assessing this impact on kava is to define the soil and plant microbiome, so we present the first known kava microbiome study. **Hypothesis:** The

kava plant associated microbiome is different between cultivars and differs from surrounding bulk soil. If validated, this hypothesis would form the basis for more extensive studies to assess the impact of associate microbiome on kava secondary metabolism. **Results:** We compared the microbial communities associated with two kava strains (*Hanakapi'ai* and *Papa 'ele 'ele*) and surrounding bulk soil. In addition, since washing of the roots is a key preparative step for indigenous kava users, we assessed the impact of this practice on the microbial community associated with roots of the *Papa kea* kava strain. **Conclusions:** Significant differences were observed between bulk soil and strains, but more importantly, significant differences in the microbiome between strains were determined. Future studies would focus on larger sample sets and replicate samples to archive statistically significant and robust differences, however, these data do indicate that cultivars indeed select their microbial ecology and root washing affects the microbiome.

CHAPTER 1

1. INTRODUCTION

1.1 Background

Piper methysticum G. Forst, meaning, "intoxicating pepper," is a shrub-like plant known predominantly as kava, or ‘Awa to the Native Hawai‘ians. It is native to Oceania, growing throughout Polynesia, Melanesia, and Micronesia [1]. The plant was domesticated ~3000 years ago in Vanuatu, and spread throughout Oceania via Austronesian colonists [2]. In the Native Hawai‘ian culture, ‘Awa is described as coming to Hawai‘i with the akua (gods) Kāne and Kanaloa. Kāne is believed to have made water appear to nurture the ‘awa crop.

Traditionally, the consumption of kava as a beverage was sacred in Pacific cultures. Indeed, in the words of Mary Kawena‘ulaokalaniahikiakaikapoliopolekawahine‘aihonuaināleilehuaapele Pukui “.....‘Awa was the food of the gods.....no religious ritual was complete without it” [3]. Offerings of ‘Awa were made to protect the health of the Hawai‘ian people, in rites of passage, to lift tabus and to both facilitate consensus-building and prepare for war or battle. Margaret Titcomb [4] summarized usages of ‘Awa: “*The ‘Awa custom is of interest in Hawai‘i because it was a sacred drink of importance in many phases of Hawai‘ian life. ... Its effect is to relax mind and body Medical kahunas (learned men) had many uses for it.....It was essential on occasions of hospitality and feasting, and as the drink of pleasure of the chiefs*”. A Hawai‘ian mele illustrating these usages is shown at left.

Various parts and preparations of ‘Awa were used medicinally in Pacific cultures. From their earliest contact with Pacific islanders, Europeans were therefore interested in kava as a medicine, first as a treatment for venereal diseases [5], and later as a sedative and treatment for anxiety [2]. A major boom in kava popularity occurred in the 1990s linked to both health-related and recreational usage for non-Pacific audiences. The most recent incarnation of the kava story is as a nutraceutical, formulated as pills and liquid

extracts, as an analogue to anti-anxiety drugs. Products are standardized to a specified concentration of kavalactones (see Figure 1), which have been extracted from kava plant material with alcohol, acetone, or water.

In the contemporary Pacific, people still drink kava. The drink is still prepared in a semi-traditional manner as a water extract served from a common bowl into smaller drinking cups (often coconut shells). The drinking protocols and associated social meanings continue to evolve. There is an awareness of the traditions associated with kava, even if little of this knowledge is incorporated into the actual way the beverage is consumed. The purpose of contemporary consumption is largely consonant with less formal consumption of earlier times, but the frequency of consumption, amount consumed, and social context of kava drinking also reflect modern shifts in perspective and social relations. Current exposure is in some cases significantly different from that in the past. As such, a review of kava's safety should examine these shifting and nuanced social dynamics, rather than reiterating past dichotomies of traditional/nontraditional consumption [6].

Contemporary kava use presents two distinct patterns of consumption: social and nutraceutical. Social kava drinking involves what is assumed to be relatively high doses (over a gram a day), with the dosage not strictly controlled or limited. Whereas kava nutraceutical consumption is of a fixed recommended daily dose (~200 mg/day) for the goal of treating a specific medical condition (usually anxiety), thus its use is personal rather than social. Traditionally, kava is mixed with water, is not extracted with another solvent, is strained by hand, and is prepared as a social drink. By contrast, nontraditional nutraceutical forms of kava are solvent-extracted, usually as part of a commercial process, and not consumed socially. In fact, extraction methods that differed from traditional water methods using solvents such as acetone or ethanol has been shown to be more efficiently in removing the kava lactones [6, 7]. Specifically, acetone extractions showed a marked increase in total kavalactones with a total percentage 89.5% compared to water extraction at 78.5% as well as a far higher percentage of all identified components at 99.4% vs. 83.7%, respectively [8]. Acetone also somewhat extracted other kavalactones not found in water due to their nonpolar nature, while ethanol extractions tended to leave out a few additional constituents, most notably flavokawain B [8], which may have anti-

carcinogenic properties [9, 10] but increased the concentration of certain kava lactones, notably yangonin and desmethoxyyangonin [8]. Thus, it should be remembered that these two forms are not identical, as they are prepared by substantially different techniques, and using different solvents. Second, kava drinking is a social activity, whereas supplement consumption is a personal activity with no inherent social dimension. Third, kava beverage is not a standardized product, whereas nutraceuticals supposedly are. A full description of traditional kava drinking and nontraditional consumption is beyond the scope of this research [2, 6], but there are some salient points for comparison when thinking about dosage, effects, and possible risk from these different consumption practices. First, the amount of kava consumed by drinkers is significantly higher than that consumed by those taking supplements. Supplement strengths range for 100 to 300 mg per dose with extracts varying from 30 to 70% kava lactones; however, most clinical studies on which safety and efficacy are based, use 100 mg doses standardized to 70% kava lactones [6, 11]. Kava drinkers will normally consume several coconut shells of kava beverage in a typical drinking session. On average, each shell contains as much more than the recommended daily dose of kavalactones used in supplement form for treating anxiety (~200 mg). The consensus of the online kava community, where the majority of non-traditional kava users gain their information, suggests that the average coconut shell serving of kava contains anywhere from 150 to 500 mg of kava lactones with no explanation as to how this concentration was determined; however, Teschke *et al.* reported a concentration of 71.5 mg of kavapyrones (kava lactones) in traditional extract made from 10 g of powdered crude kava in 100 mL of water, which calculates to a daily dose of 210 mg per 300 mL [12]. A night's dose of kavalactones from drinking kava (5-10 shells) could easily be in the range of 1.0-2.5 grams. This is considered high, even according to multiple kava online forums/websites that claim the Kava Committee of the American Herbal Products Association (AHPA) recommended no more than 300 mg/day and the Federal Drug Administration (FDA) recommendation of 290 mg/day, though these claims could not be verified and further sheds light on the challenges of kava safety and dosing.

It is difficult to accurately determine the number of people who are consuming kava, the amount of kava they are taking, and the frequency with which they use it;

especially since sales and import data is often proprietary [13]. Global use of kava supplements was certainly substantially lower than it was in 2001, prior to bans instituted by several countries due to concerns about liver toxicity [7, 14-20]. However supplement use in the US continues with over 50% of Americans taking some form of dietary supplement, while consumption of kava, as a social beverage, seems to be increasing [21-29]. Data on the amount of kava produced and exported are not accurate, sales figures for kava products are not widely available, and it is difficult to estimate number of users since both the production and consumption sides of the commodity chain are fragmented. However, one kava beverage company, Taki Mai, reported that Fiji earned over \$20 million in kava exports between 2012 and 2014 and estimates the exports to the U.S. alone should reach the \$15 million estimating over 100 kava bars in existence. Fiji is so sure that the market is growing, that the Ministry of Agriculture initiated the Kava Act of 2016 as they plan to increase growing of kava to meet the demand with Fiji currently producing approximately 4000 tonnes of kava on 1300 hectares.

Kava is grown in more than six different island nations in the Pacific and in the wider Pacific Islander diaspora. It is consumed locally and exported to the United States for manufacture into nutraceuticals. Fiji, Vanuatu, Samoa, and Tonga are primary kava-exporting countries[30]. Export statistics from the producer nations give a partial glimpse of consumption, but they are not widely available or reliable. The decentralized, minimally regulated nature of kava's commodity chain contributes to this uncertainty. With respect to supplement use, several companies produce kava supplements (and other products such as kava skin creams), primarily for the US market. There is the potential for this market to dramatically increase, following a 2014 court decision in Germany that overturned the ban on kava products in that country. Changes such as this to the regulatory frameworks in which kava is embedded could quickly affect the availability of these products.

Kava consumption in the US has expanded through supplement availability and most recently through the proliferation in kava bars. In the Pacific, there have also been changes in the pattern of kava consumption. Migration within the region has brought kava drinking to places where it was not previously a tradition (e.g., Kiribati, New Caledonia, the Solomon Islands, and New Zealand; see [6, 31]). In addition, changing

social practices in societies for which kava drinking is a tradition may be leading to increased consumption. For example, more women are drinking kava in the Pacific than in previous decades, as kava consumption in the Pacific was generally considered taboo for women. Though some cultures allowed kava to be served by women, the activity was mostly kept separated to include not engaging in sex with women after consuming kava [2]. 'Awa use by women in traditional Hawai'ian culture is known; however, it was mainly allowed by ali'i class women under rare ceremonial conditions and rarely in the presence of men [4, 32]. 'Awa was even offered to female deities, especially Pele who was fond of *hiwa* and *mō 'ī* varieties [32, 33] and is documented in chants to her by females of her clan [32]. On the other hand, taboos of 'Awa use is also represented with kapus being placed on females of reproductive age due to concerns of premature birth and other negative effects [32, 34]. Overall, the increased acceptance of women drinkers in addition to other factors indicate there is more demand for kava for general consumption, and more kava consumed in those drinking sessions, as well as an increase in growing kava [35]. In light of these points, a review of the potential toxicity of kava is timely.

The known active ingredients in kava are the kavalactones. Eighteen of these have been identified, but only six of them: methysticin, dihydromethysticin, kawain, dihydrokawain, desmethoxyyangonin and yangonin, have been the focus of kava studies as they make up 96% of organic extracts [36]. See Figure 1. for structures of major kava components. However, kava extractions contain a variety of other non-lactone compounds, which may be responsible for the pharmacological benefits and potential toxicity [8, 36]. The activity of kava may be the result of one of these non-lactone compounds or a synergy of several or all components found in kava. In fact, studies on RBL2H3 mast cells (Rat Basophilic Leukemia subtype 2H3, purchased from ATCC® CRL-2256), showed that traditional aqueous kava extracts elicited strong calcium responses not seen in individual or combined purified kava lactones, specifically methysticin, dihydromethysticin and kawain [37]. Furthermore, traditional aqueous kava extracts demonstrated mast cell degranulation whereas purified lactones did not [37].

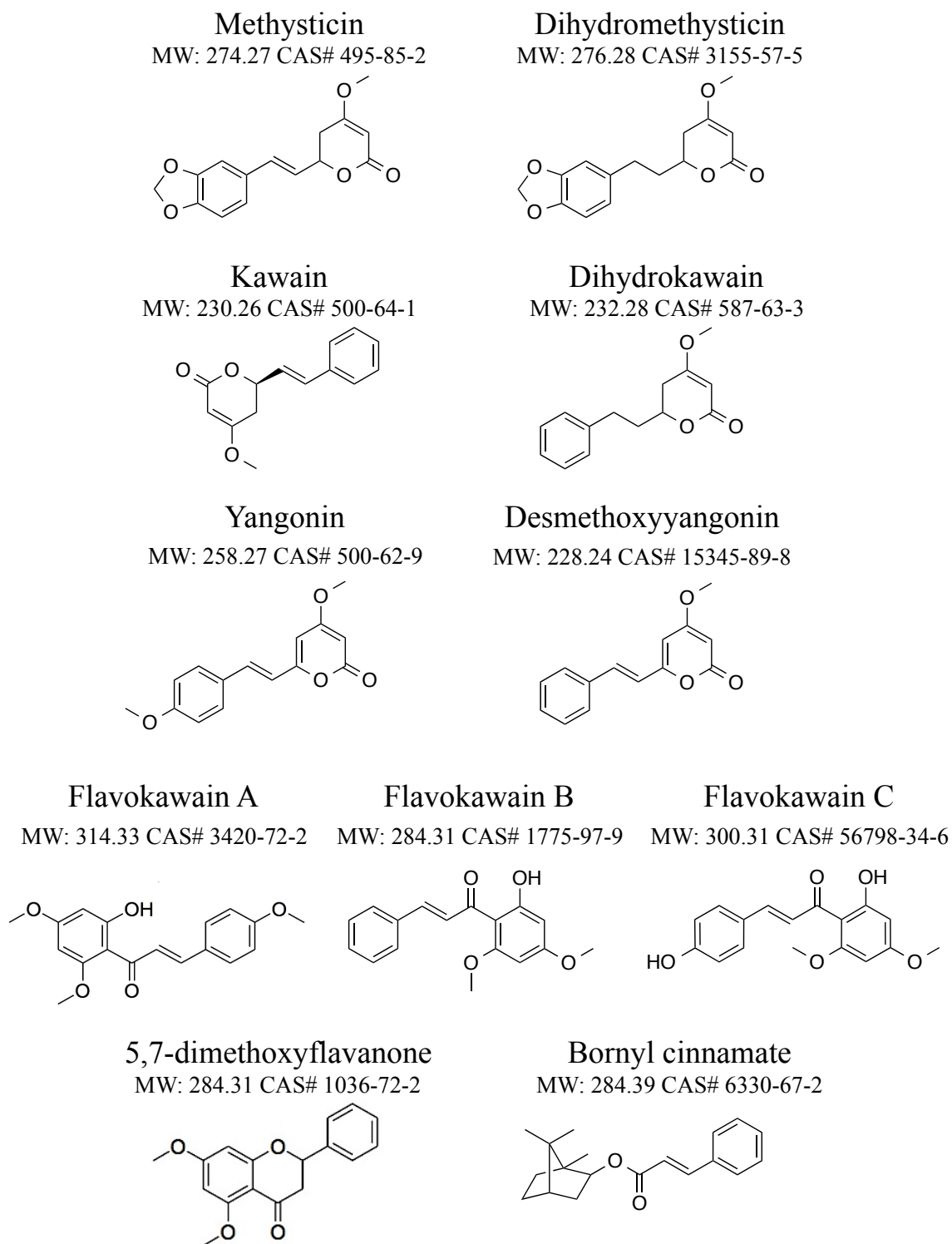


Figure 1. Kava constituents discussed in the text. [38].

A focus on kavalactones may neglect other important compounds in kava that can direct cellular responses. Additional kava components include the dihydrochalcones (flavokawains A, B and C), 5,7-dimethoxyflavanone, cinnamic acid bornyl ester as well as tentatively identified compounds classified as phenolics, flavanones, fatty acids and a chalcone, specifically 2,5,8-trimethyl-1-naphthol, 5-methyl-1-phenylhexene-3-yn-5-ol, 8,11-octadecadienoic acid-methyl ester, 5,7-(OH)₂-4'-one-6,8-dimethylflavanone, 7-dimethoxyflavanone-5-hydroxy-4' and pinostrobin chalcone [8]. Cinnamic acid has been shown to activate the mast cell calcium channel TRPA1 and has been associated with contact dermatitis [39-41] while the pinostrobin chalcone can act as a stimulatory or inhibitory molecule on mast cells [42]. Equally important, the type of extraction solvent used has a marked effect on the ratios of these compounds, as well as the kavalactones, with some compounds not being extracted at all [8].

Though studies have focused on the kavalactones being the primary components of kava extractions, correctly since they have demonstrated a mechanistic connection to gamma amino butyric acid (GABA) receptors, the chemical complexity of kava extracts suggests that a kavalactone-centric approach may (1) underestimate the complexity of and (2) not provide a mechanism for some of the non-GABA based medicinal effects. A comprehensive understanding of 'Awa chemistry is of importance in assessing the future of kava exposure in both Pacific and global populations. This generates two key considerations. First, the type of extraction to be characterized has important implications. There is a tension between fidelity to the traditional aqueous extractions of primarily root samples, and the need to analyze organic extracts of aerial and root powders that are the major nutraceutical forms of commercialized 'Awa. There is good evidence that kava toxicity and efficacy are linked to extraction method. Since both traditional and commercial/organic extracts are public health issues, both need to be examined comprehensively.

Kava plants are likely to contain a diverse secondary metabolome, with hundreds of compounds that can impact the physiological responses of human cells and tissues [8, 36, 43-45]. The focus of the 'Awa field upon the kavalactones is linked to the strong likelihood that these compounds' ligation of central nervous system (CNS) GABA receptors is responsible for the relaxant and anxiolytic effects of the drink and its

supplements [46, 47]. However, the physiological (and possibly pathophysiological) effects of kava may be underestimated by a unilateral focus upon the kavalactones. The secondary metabolome of *Cannabis sativa* provides an analogy here. For decades the primary focus of the field, the marijuana growing community, and medicinal marijuana proponents has been on the major cannabinoid compounds Δ^9 -THC, cannabidiol and cannabinol. These are indeed the main CNS-active components but they and their derivatives comprise ~7 of the >400 known bioactive molecules in *Cannabis sativa*. Indeed, until the so-called 'entourage' of terpenes, alkaloids, etc., was factored into cannabinoid pharmacology [48, 49], our understanding of its mechanisms and breadth of effect was severely limited. Similarly, the 'Awa field may now benefit from examination of the *Piper methysticum* 'entourage'.

A more in depth published literature review (Contemporary Pacific and Western perspectives on 'Awa (*Piper methysticum*) toxicology. Showman AF, Baker JD, Linares C, Naeole CK, Borris R, Johnston E, Konanui J, Turner H. Fitoterapia. 2015 Jan; 100:56-67. Review. PMID: 25464054) is located in Appendix I.

1.2. Why study kava?

Studying kava gives Western pharmaceutical an opportunity to understand the traditional indigenous practice. These indigenous practices include relaxation, conflict resolution, relationship-building, psycho-spiritual/ritual and medicinal uses. In Hawai'ian culture, native plants like 'Awa is a part of their family, and the sharing of that plant symbolizes a contract between them and their ancestors. Thus anything said during the social practice is contractual and their ancestors make them accountable [50]. Medicinally, different strains are selected for different uses based on their effects and their mana. For example, nene is used for mind and body relaxation and given to those suffering back and muscle pain and to reduce stress while *hiwa* stains, (meaning black, night, magestical), such as *Papa 'ele 'ele* is given to bring intuitive dreams [50]. Western use of kava has also been for anxiety and anti-inflammatory conditions, but kava may also have anti-carcinogenic benefits.

Further investigation into the potential human health issues (possible hepatotoxicity, kava dermatopathy). Despite the link to kava and liver toxicity demonstrated *in vivo* and *in vitro*, in the history of Western kava use, toxicity is still considered relatively rare. Only a fraction of the handful of cases reviewed for liver toxicity could be, with any certainty, linked to kava consumption and most of those involved the co-ingestion of other medications/supplements [14, 51]. That means the incident rate of liver toxicity due to kava is one in 60-125 million patients [18]. For Pacific traditional users, despite the much higher kavalactone exposure, 'Awa liver toxicity is either unheard of or unreported. Nevertheless, in rural areas of the Pacific, where hepatitis is endemic, liver disease that may be caused by kava consumption may be masked and reported as other causes [15]. It is difficult to say with any sense of accuracy since there is a shortage of epidemiology and public health data in Pacific populations who habitually use kava. Much of the linked toxicity has been associated with type of extract; nutraceutical forms of kava are generally extracted through the use of organic solvents such as acetone or alcohol to increase the amount of kava lactones retained. Traditionally, water extracts have been used for making of the social and medicinal drink; however, alcohol is used in making tinctures from 'Awa to increase its potency by soaking the dried 'Awa roots from moon to moon in an alcohol concentration of 40% or higher. These tinctures are often used to treat toothaches and other mouth problems [50].

Kava is of agro-economic importance in Hawai'i and the Pacific, and the growing community needs scientific support. With the lifting of the European kava ban and increased growth of non-traditional kava drinking establishments in the mainland, USA, kava has great potential to become a cash-crop for Hawai'i, as well as the rest of the Pacific islands. For some time, the economy of Hawai'i has needed to diversify the existing industrial complex that has been bases for generations. 'Awa has the potential to be this important economic diversification; however, in doing so, it is of importance that safety, efficacy and quality control be maintained and these can be supported by the scientific community working closely with local growers.

1.3 Kava Knowledge Gaps

We lack a mechanistic understanding of active compounds of kava and what these compounds target in the human body, physiological and pathophysiological effects. Furthermore, we need to understand the breadth of bioactive compounds in kava, as the current focus has only been on a limited number of kavalactones. Finally, the scientific community has not provided information that supports industry in growing and testing (standardization, toxicology, provenance) of kava for consumption.

1.4. Objectives Overview

In this work, I hope to **(1)** identify the physiological effects of kava exposure in cells of the immune system and **(2)** provide the first pilot evaluation of the kava plant associated microbiome.

(1) Identification of physiological effects of kava exposure in cells of the immune system. *Rationale:* The efficacy and possible toxicity of kava may reflect additional biological targets in addition to GABA receptors in the CNS. ***Hypothesis:*** Immune system cells and their calcium signaling channels (e.g. the TRP family that have been shown to react to plant secondary metabolites from diverse sources) may be novel targets for kava constituents. If validated, this hypothesis would illuminate new understandings of the therapeutic potential of kava, as well as the indigenous medicine indications of the drink.

(2) Pilot evaluation of the kava plant associated microbiome. *Rationale:* Production of these secondary metabolites that regulate human physiology are heavily affected by changes in the physical and biological environment of the whole plant, including the plant's associated microbial community. The first step towards assessing this impact on kava is to define the soil and plant microbiome, so we present the first known kava microbiome study. ***Hypothesis:*** The kava plant associated microbiome is different between cultivars and differs from surrounding bulk soil. If validated, this hypothesis would form the basis for more extensive studies to assess the impact of associated microbiome on kava secondary metabolism.

CHAPTER 2:

2. INTRODUCTION

In this chapter, we suggest that some of the efficacy and toxicity of kava may additionally be caused by TRP and I_{CRAC} cellular responses via the influx and efflux of calcium ions. Here, the population based, and single cell (confocal) analysis of kava components (crude extracts, LCMS fractions and synthetic compounds) using fluorescent calcium-sensitive dyes (Fluo-4) and electrophysiology recordings of calcium-selective cation channel were used to determine activity induced by kava extracts, fractions and synthetic compounds. Results suggest that the physiological and pathophysiological effects of kava is more complex than just CNS and GABA induced responses by the kava lactones and may be the result of secondary kava metabolites. These findings have been published in a peer-reviewed paper, which is reproduced below:

Differential regulation of calcium signalling pathways by components of *Piper methysticum* ('Awa)

L.M.N. Shimoda, A. Showman, J. D. Baker, I. Lange, D. L. Koomoa, A. J. Stokes, R. P. Borris and H. Turner (2015). *Phytother. Res.* 29: 582-590.

2.1. Abstract

Kava is a soporific, anxiolytic and relaxant in widespread ritual and recreational use throughout the Pacific. Traditional uses of kava by indigenous Pacific Island peoples reflect a complex pharmacopeia, centered on GABA-ergic effects of the well-characterized kavalactones. However, peripheral effects of kava suggest active components other than the CNS-targeted kavalactones. We have previously shown that immunocytes exhibit calcium mobilization in response to traditionally prepared kava extracts, and that the kavalactones do not induce these calcium responses. Here, we characterize the complex calcium-mobilizing activity of traditionally prepared and partially HPLC-purified kava extracts, noting induction of both calcium entry and store release pathways. Kava components activate intracellular store depletion of thapsigargin-sensitive and -insensitive stores that are coupled to the calcium release activated (CRAC) current, and cause calcium entry through non-store-operated pathways. Together with the pepper-like potency reported by kava users, these studies lead us to hypothesize that kava extracts contain one or more ligands for the transient receptor potential (TRP) family of ion channels. Indeed, TRP-like conductances are observed in kava-treated cells under patch clamp. Thus TRP-mediated cellular effects may be responsible for some of the reported pharmacology of kava.

2.2. Introduction

A drink made from varying preparations of ground rhizome and root from *Piper methysticum* plays a key role in Pacific island ritual and social interactions [52-56]. Various, the beverages 'Awa, sakau, ava, kava-kava and yaqona (Hawai'i, Micronesia, Tonga and the Marquesas, Fiji) play a role in traditional decision-making processes, and in the building of relationships and consensus in small island contexts [54, 57, 58]. Contemporary recreational use has outpaced the degree to which traditional practitioners can guide kava preparation and cultivation, and has extended kava's impact to a global recreational and nutraceutical audience. This globalization of kava brings new

perspectives to its study, placing Western drug discovery and toxicology/efficacy studies alongside opportunities to explore the mechanistic bases for kava's actions in a manner informed by indigenous knowledge [1, 15, 19, 59, 60].

Kava has a broad indigenous pharmacology that encompasses both CNS-centered and peripheral effects [61]. The CNS-centered effects of kava are the most highlighted in sacramental and ritual kava drinking, recreational use and contemporary nutraceutical marketing campaigns. These are the sedative and calming effects which, in the nutraceutical industry, are promoted as treatments for stress, anxiety and depression, often portrayed as 'natural' analogues of anxiolytic and antidepressant pharmaceuticals [55, 59, 62]. The candidate bioactive secondary metabolites in kava for these CNS-centered effects are the kavalactones [46, 58, 60, 63-65]. These compounds include kavain, methysticin and dihydromethysticin (refer to Figure 1.) and are putative ligands for GABA receptors [46, 47]. Peripherally, kava (as beverage, root or various plant parts) is indicated in traditional Pacific medicine for urogenital conditions (gonorrhoea infections, chronic cystitis and difficulty urinating), reproductive and women's health (for menstrual problems and dysmenorrhea, to facilitate delivery, to stimulate milk production, its leaves as an abortifacient and contraceptive), gastrointestinal distress, respiratory ailments (asthma, coughs and tuberculosis), skin diseases and topical wounds, and as an analgesic [54, 55, 57-59]. Significant subtlety and nuance attend the precise strain, plant component and preparative method to be used [54, 55, 57-59]. These data suggest active components in kava that extend beyond the GABA-ergic kavalactones, and that may be sufficiently varying with strain, component and preparative method to underlie the complexity that is evident in the traditional pharmacopeia [8, 36, 43-45].

The premise of the current study is that the broad peripheral effects of kava, together with untapped potential medicinal efficacy and concomitant toxicology concerns, create a need to understand the cellular impact of both kavalactones and non-kavalactone components of the kava drink [15, 19, 59-61, 66-69]. There have been few reports of the cellular signalling pathways regulated by kava components other than kavalactones. Our previous studies suggest that at least one major target in peripheral cells is the mobilization of intracellular free calcium, a signal of sufficient magnitude and complexity to engender complex downstream effects at the organ and tissue level [37].

Moreover, the reported presence of bioactive secondary metabolites in other *Piper* spp. that affect the gating of TRP channels (sensors for compounds such as capsaicin, allicin, vanillin, gingerol, cinnamaldehyde, menthol and others) lead us to hypothesize that kava may contain TRP-active components. Some of these (e.g. cinnamaldehyde) have been reported in kava extracts [8, 70-73]. Here we perform a detailed analysis of the calcium-mobilizing activity of both raw and fractionated kava extracts, adhering to traditional extraction methods informed by Hawai'ian kūpuna as a basis for starting analytes.

2.3. Methods and Materials

2.3.1. Cell culture:

RBL2H3 (Rat Basophilic Leukemia cell line subtype 2H3) from ATCC (CRL-2256) [74] were grown at 37 °C, 5% CO₂, in 95% humidity in Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA) with 10% heat-inactivated fetal bovine serum (Mediatech) and 2mM glutamine.

2.3.2. Chemicals:

General chemicals were from VWR (West Chester, PA). Thapsigargin and ionomycin were from Calbiochem (Gibbstown, NJ).

2.3.3. Kava extract purification:

Powdered kava root was obtained from *P. methysticum* 'Awa strains *Papa kea*, *Papa 'ele 'ele* and *Hanakāpi'ai* grown in Pepe'ekeo (19° 50' 12" N 155° 6' 19" W) Hilo, Hawai'i by Mr. Edward Johnston (Association of Hawai'ian 'Awa, August 2013). *P. methysticum* G. Forst voucher specimens are held by the Bishop Museum, Honolulu, Hawai'i. Annotated specimens and germplasm of the Hawai'ian cultivars traditionally named here are documented in the archives of the Association of Hawai'ian 'Awa, Hilo, Hawai'i [1]. Roots were harvested, washed (kūpuna Jerry Konanui, Pahoa, HI) and ground into a fine paste. Standardized water-based extractions were performed to generate a 1% (w/v) suspension, with gentle agitation (magnetic stirrer) for 5 min preceding filtration through a fine fabric mesh. This protocol reproduces, as closely as possible, the traditional method of extraction that is currently used in the Pacific [1, 37]. Commercial dry powder preparations of *Mahakea* (Hawai'ian Kava Center, Honolulu,

HI) and *Kū makua* (Maui 'Awa Company, Lahaina, HI) were processed similarly. Sequential chromatography approaches for commercial 'Awa extracts generated 8 subsequent batches of test analyte. Data from sequential fraction of Batch 1 (HPLC analysed using Shimadzu Sunrise C18 column, 10 x 2 50 mm, 5 micron particles, eluted with acetonitrile:water:formic acid (400:600:1) @ 3 mL/min) are presented here.

2.3.4. Imaging:

Bright field and fluorescence imaging of cells in MatTek dishes (50,000 cells per cm²) were performed on a Nikon Ti Eclipse C1 epi-fluorescence and confocal microscopy system, equipped with a heated stage. Available laser lines in FITC, TxRed and Cy5 were supplied by a 488-nm 10-mW solid state laser, a 561-nm 10-mW diode pump solid state (DPSS) laser and a 638-nm 10-mW modulated diode laser. Each z disc (optical section) was 150 nm. Pinhole size for all images was 60 microns. Images were analysed in NIS Elements (Nikon, Melville, NY).

2.3.5. Calcium assay (bulk method):

RBL2H3 were washed and incubated with 2.0-μM Fluo-4 for 30 min at 37 °C in a standard modified Ringer's solution of the following composition (in mM): NaCl 145, KCl 2.8, CsCl 10, CaCl₂ 10, MgCl₂ 2, glucose 10, Hepes·NaOH 10, pH7.4, 330mOsm. Cells were transferred to 96-well plates at 50 000 cells/well and stimulated as indicated. Calcium signals were acquired using a Flexstation 3 (Molecular Devices, Sunnydale, USA). Data was analysed using SoftMax® Pro 5 (Molecular Devices). Where indicated, nominally calcium-free external conditions were achieved by the preparation of 0mM CaCl₂ Ringer solution containing 1mM EGTA.

2.3.6. Calcium assay (single cell method):

RBL2H3 were plated on glass coverslip dishes (MatTek, Ashland, MA) and incubated with 1-μM Fluo-4 for 30 min at 37 °C in a standard modified Ringer's solution as described above. After washing, cells were stimulated as indicated on a 37 °C heated stage. Calcium signals were acquired using a Nikon Ti Eclipse confocal microscopy system, using EZ C1 software for acquisition and NIS Elements software (Nikon) for analysis. Where indicated, nominally calcium-free external conditions (indicated as ~0

mM) were achieved by the preparation of 0 mM added CaCl_2 Ringer solution containing 1mM EGTA.

2.3.7. Electrophysiology:

For patch-clamp experiments, cells were grown on cell-culture glass-bottom dishes (Cellview, Greiner Bio-One, Germany) and kept in a standard modified Ringer's solution of the following composition (in mM): NaCl 130, CsCl 2.8, CaCl_2 20, MgCl_2 2, glucose 11, Hepes·NaOH 10, pH7.3. Intracellular pipette-filling solutions contained (in mM): Cs-glutamate 140, NaCl 8, MgCl_2 3, Cs-BAPTA 10, pH7.3 adjusted with CsOH. In order to prevent passive store depletion, CaCl_2 was added, and free calcium was clamped to ~177nM free, calculated using Webmaxc Standard (<http://web.stanford.edu/~cpatton/webmaxcS.htm>). Agonists were dissolved in the standard extracellular solution, containing 10mM calcium. Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21– 25 °C. Current recordings were acquired by patch-clamp amplifier system EPC-10-USB (HEKA, Lambrecht, Germany). Glass pipettes had resistances between 2.5 and 3.5M Ω after filling with the standard intracellular solution. Immediately following establishment of the whole-cell configuration voltage ramps of 50-ms duration spanning the voltage range of -100 to +100mV were delivered from a holding potential of 0mV at a rate of 0.5Hz over a period of 500 to 800 s. All voltages were corrected for a liquid junction potential of 10mV between external and internal solutions. Currents were filtered at 2.9kHz and digitized at 10-kHz intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the capacitance compensation of the EPC-10. For analysis, ramps were digitally filtered at 2kHz. Currents were normalized to the current obtained before development of currents.

2.3.8. Analysis:

Results are shown as the mean + standard deviation. Statistical significance was determined based on ANOVA or Student's t-test where appropriate. Adjacent to data points in the respective graphs, significant differences were recorded as follows: single asterisk, $p < 0.05$; double asterisk, $p < 0.01$; triple asterisk, $p < 0.001$; no symbol, $p > 0.05$.

Experiments are n of 3– 10. Where indicated the integral Area Under the Curve (AUC) was calculated using GraphPad Prism.

2.4. Results

2.4.1. Kava extract-induced conductances contain store-operated and TRP-like components

In a previous study we have shown that kava extracts initiate striking elevations in intracellular free calcium in a model immune system cell, the RBL2H3 basophil [37, 74]. This action on calcium signalling is independent of the kavalactones, commonly viewed as the major 'active' components of kava [37]. In the current study, we tested the hypothesis that kava extracts would include compounds active at members of the TRP calcium permeant non-selective cation channel family. RBL2H3 are used here as a convenient model system because they co-express a range of calcium entry mechanisms including store operated calcium entry via I_{CRAC} , and various TRPs [40, 75-80]. Kava extracts from the Hawai'ian *Mahakea* strain were applied to RBL2H3 in a single cell, whole cell patch clamp configuration. Across the cell population, three discrete kava-induced conductances were observed. Figure 2. (A and B) shows a TRPV1-like current, with a 0-mV reversal potential, attaining inward current amplitudes of > 10 pA/pF. These currents were present in approximately 30% of cells assayed. Figure 2. (C and D) shows a TRPM2-like current, again developing to large (> 20 pA/pF) magnitudes and reversing at 0mV. TRPM2-like currents were observed in approximately 18% of the cells assayed. Finally, Figure 2. (E and F) shows the development of a small conductance with the distinctive signature associated with I_{CRAC} ; developing to 1– 2pA/pF inward amplitude and reversing at approximately +40mV. This conductance was also observed in approximately 20% of the cells assayed.

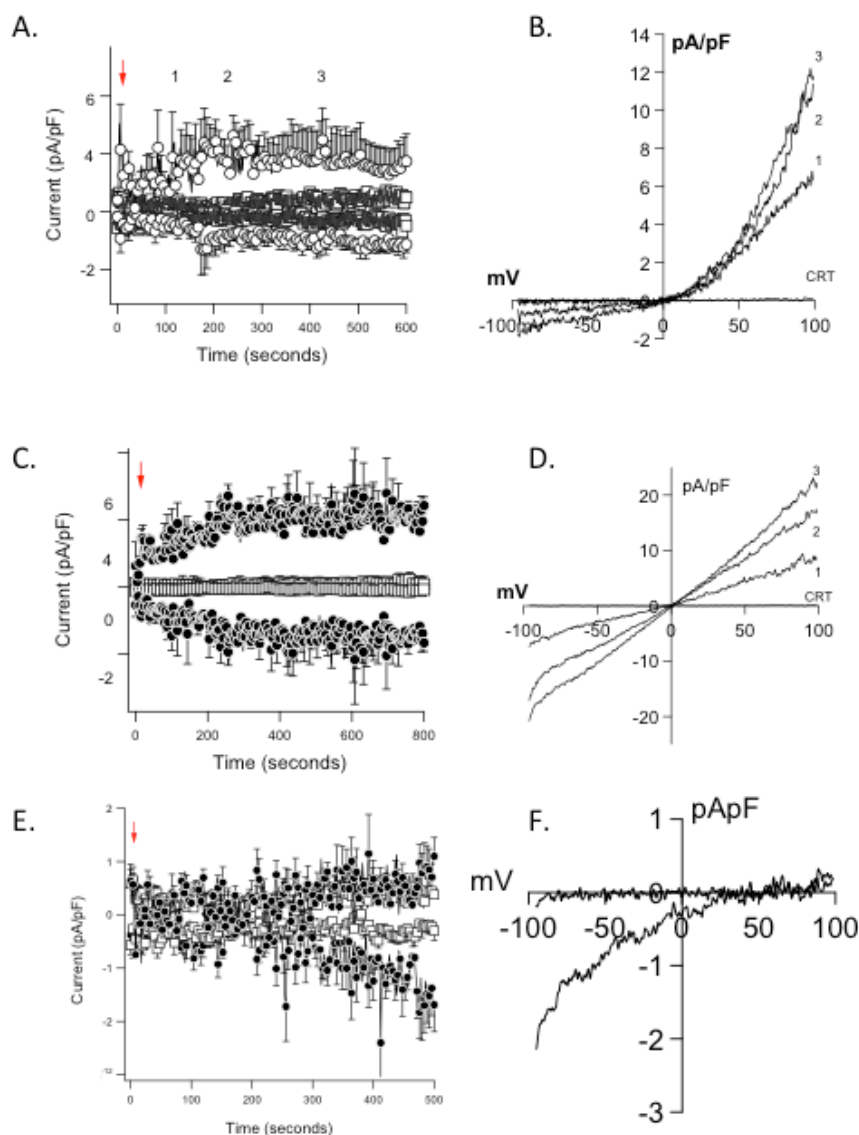


Figure 2. Whole cell patch clamp analysis of kava extract-induced conductances in RBL2H3 cells.

RBL2H3 were analysed in the whole cell configuration with applied 1% aqueous kava extracts (*Mahakea*) prepared as described and applied in internal solution over the indicated time course. Three types of conductance were observed repeatedly in the cell population ($n=21$ cells). Examples of each of these conductance types are shown here. **A.**, **C.**, **E.** Inward and outward current development over time. **B.**, **D.**, **F.** Current/voltage (I/V) relationships. **A.**, **B.** Development of TRPV-like current after application of kava extract ($n=7/21$). **C.**, **D.** Development of TRPM-like current after application of kava extract ($n=2/21$). **E.**, **F.** Development of I_{CRAC} -like current after application of kava extract ($n=4/21$). CRT; control. External solution: 20mM $CaCl_2$, 2mM $MgCl_2$, 130mM NaCl, 2.8mM CsCl, 10mM NaOH-HEPES, 11mM glucose. Internal solution: 140mM Cs-Glut, 8mM NaCl, 3mM $MgCl_2$, 10mM CsOH-HEPES, 10mM BAPTA, 4.3mM $CaCl_2$.

2.4.2. Kava extracts prepared in the traditional manner cause calcium influx and release from intracellular stores

We asked whether the *Mahakea*-induced conductances in Figure 2. translated to significant calcium influx responses, and whether store release was involved. Initial experiments with complete *Mahakea* samples suggested that both release and influx responses were visible in bulk calcium assays [37]. We fractionated complete extracts into 9 sub-fractions, using C18 columns and acetonitrile elution, in order to dissect these responses. Figure 3.A. shows that, like complete extracts, early fractions (F1 and F2) induce a rapid elevation in intracellular Fluo-4 fluorescence, upon which is superimposed a developing calcium influx response that is similar in character to those observed with stimuli such as antigen and thapsigargin in these cells. We interpret these data such that F1 and F2 may contain autofluorescent compounds that initially artificially elevate the baseline (2–10 s after addition) [81] and that a conventional calcium influx pathway is then activated. Notably, sub-fractions 3–9 contain calcium-mobilizing activity that is not complicated by the presence of significant autofluorescence. Figure 3.B. shows that, in nominally calcium free media, the initial (probably autofluorescence-based) elevation in signal caused by F1 and F2 is not followed by marked influx. However, while some sub-fractions contained an apparent and very minor release response (F3–9), the bulk assay system could not definitively address the issue of store release.

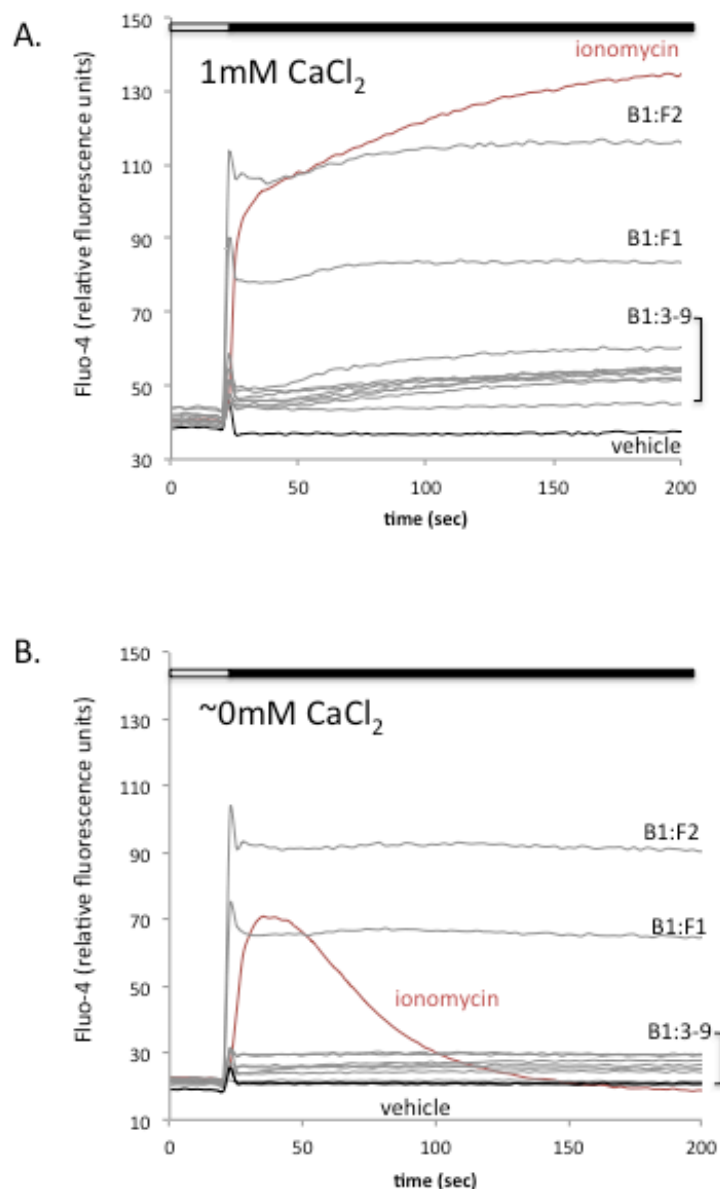


Figure 3. Apparent calcium influx and store release in kava-treated RBL2H3 cells.

RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, traces are mean of triplicate wells) for kava extract (*Mahakea* starting material) fraction-induced calcium responses. Experiments were performed in 1mM CaCl₂ and nominally calcium free (~0mM CaCl₂ with 1mM EGTA) external solutions (A. and B., respectively). Open bar indicates baseline period prior to stimulus addition. Black bar represents period of exposure to stimulus. Responses to vehicle (black trace), ionomycin (500 nM, red trace) and fractions (F1–9) from the batch 1 (B1) HPLC protocol (HPLC—Shimadzu Sunrise C18 column, 10 x 250mm, 5 micron particles, eluted w/acetonitrile:water:formic acid (400:600:1) @ 3mL/min), are shown.

In Figure 4. we used a single cell calcium imaging assay system to address this point. This system is free of the fluorescence artifacts [81] that we note in the bulk assay. Figure 4. shows averaged traces from >50 individual cells loaded with Fluo-4 and to which *Mahakea* samples were added using a microapplicator system. In nominally calcium free conditions we noted release responses above baseline variations (green trace) and the areas under the curve (AUC) calculated for these release responses were significantly different ($p < 0.01$) from vehicle-treated cells. These data are definitive for the presence of calcium store release-inducing activity in *Mahakea* samples.

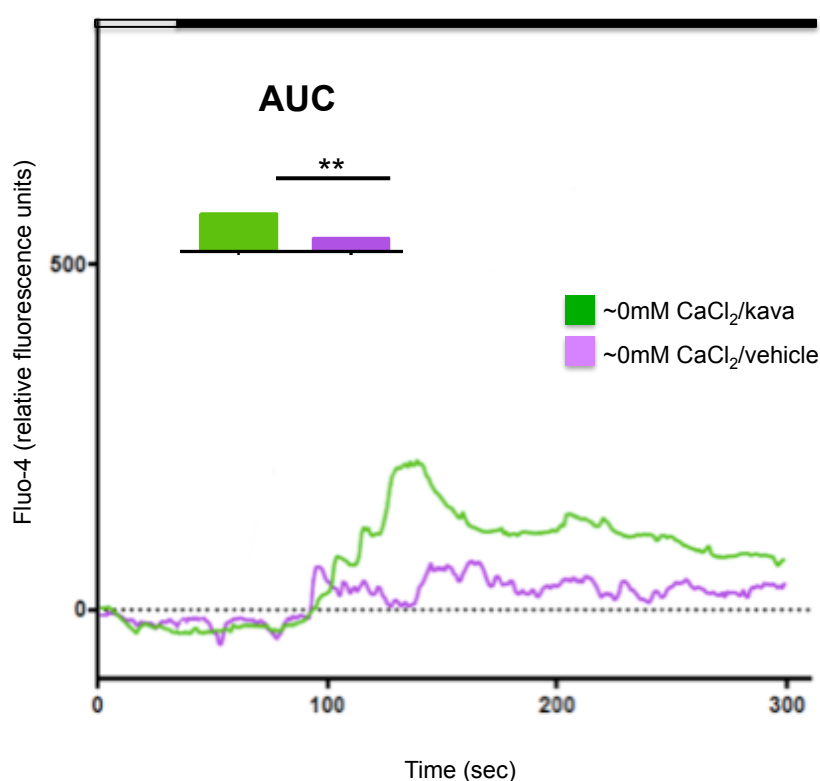


Figure 4. Single cell calcium assay of kava extract-induced release and influx responses in RBL2H3.

Single cell calcium imaging was performed in Fluo-4 loaded cells stimulated with 1% aqueous 'Awa extracts (*Mahakea*). Black bar represents period of exposure to stimulus. Single z discs (150 nm vertical step) were analyzed by drawing a whole cell region of interest (ROI) and assessing the whole cell averaged Fluo-4 intensity over time. Green trace. Averaged release response to extract from 23 cells in nominally calcium free buffer. Violet trace. Averaged release response to vehicle from 23 cells in nominally calcium free buffer. **Inset:** Area under the curve analysis (AUC) for indicated data sets.

2.4.3. Kava constituents release calcium from thapsigargin-sensitive and -insensitive intracellular stores

We further characterized the influx and release responses induced by *Mahakea* samples. Figure 5.A. shows that influx responses induced by *Mahakea* samples are not additively increasing those induced by thapsigargin (i.e. I_{CRAC} induction). Calcium add-back experiments (Figure 5.B.) showed that, at the population level, kava samples were able to additively enhance influx responses over and above those initiated by thapsigargin-mediated store depletion via I_{CRAC} . Thus kava components are recruiting non-CRAC channels. At the level of store release (Figure 5.C.), we noted that depletion of thapsigargin sensitive stores did not prevent subsequent release responses initiated by *Mahakea* samples, indicating that these stores are non-overlapping. This was also true when stimuli were added in the reciprocal sequence and for Inositol (1,4,5) trisphosphate (Ins (1,4,5) P₃) sensitive store compartments (not shown). These data suggest that intracellular calcium stores sensitive to kava components are not completely overlapping with the SERCA or Ins (1,4,5) P₃ sensitive stores.

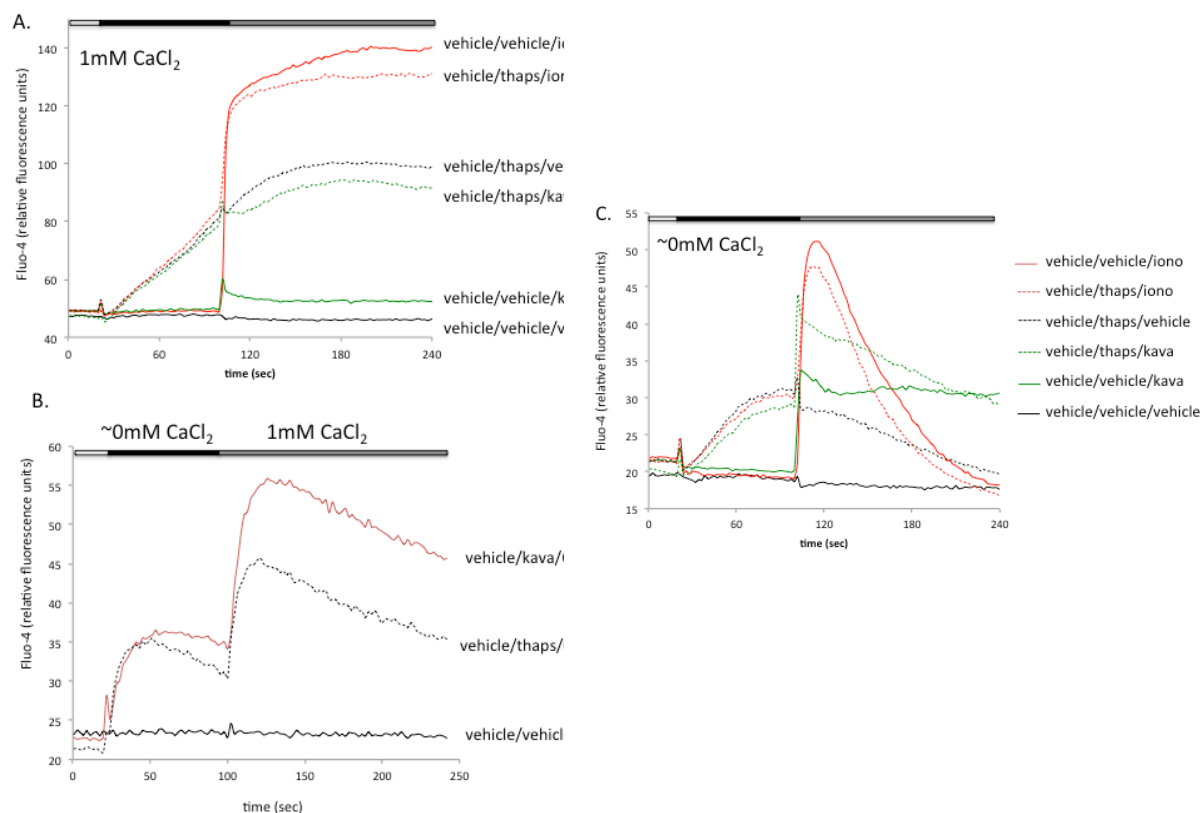


Figure 5. Dissection of calcium store release responses induced by kava and the SERCA inhibitor Thapsigargin. A–C.

A–C. RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, traces are mean of triplicate wells) for 1% aqueous kava extract (*Mahakea* starting material) induced calcium responses in 1mM external calcium (**A.**, **B.**) or nominally calcium free conditions (**C.**). Each experiment had three phases: Open bar represents baseline establishment. Black bar represents duration of primary stimulus application. Grey bar represents duration of secondary stimulus application. The sequence of baseline/primary/secondary stimuli is shown at right of each trace or in legend. Thapsigargin and ionomycin were used at 500 nM. **B.** Calcium add-back experiment protocol. Baseline and primary stimulus (1% aqueous kava application, 500-nM ionomycin) application were performed in nominally calcium free external conditions (open and black bars). Calcium was then resupplied to a concentration of 1mM in the third phase of the experiment (grey bar).

2.4.4. Traditionally prepared kava extracts contain chemically separable store-operated and non-store-operated influx inducing components

The complete secondary metabolome of kava has not been defined, and it is therefore difficult to assign likely candidate molecules to the calcium-mobilizing responses described here. As a first step in this process we asked whether: (i) the activities that initiate release and influx (of the store-operated and non-store operated types) were chemically separable, and (ii) whether different kava chemotypes/cultivars displayed differential abilities to mobilize calcium by any of these pathways. Figure 6.A.–E. shows various *Mahakea* sub-fractions analysed for their abilities to initiate release and influx. These data are summarized within Table 1, which shows that the intensity of release and influx responses is chemically separable. Moreover, when we differentiated between store-operated and non-store-operated calcium influx (SOCl and non-SOCl, i.e. that occurring putatively via I_{CRAC} and TRP-type channels, respectively), we saw a further level of differentiation. Non-SOCl was defined as that occurring additively to the influx initiated by store depletion using thapsigargin (Figure 5). There are also clearly (as in Figure 3) issues with fractions where there may be fluorescence background.

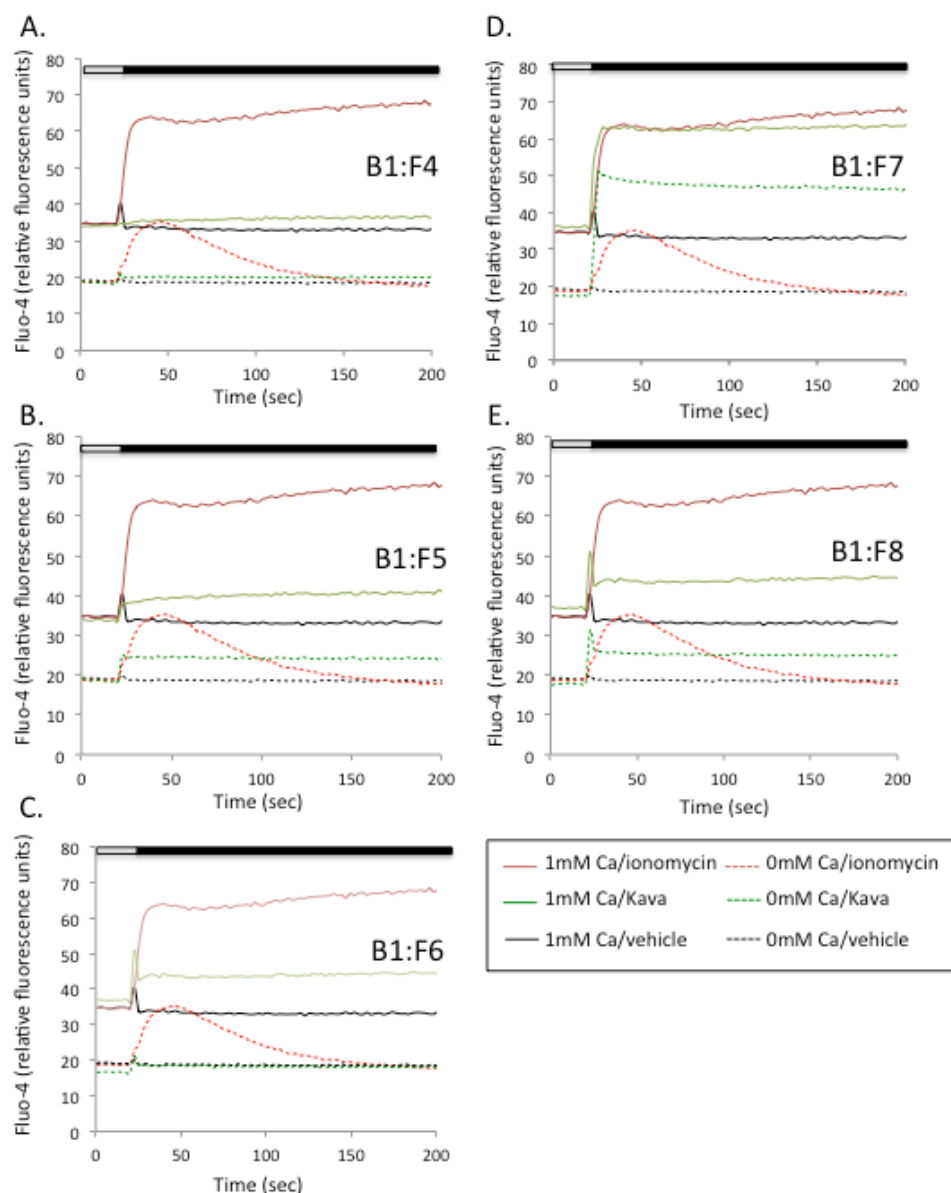


Figure 6. Fractionation of aqueous kava extracts differentially preserves calcium release and influx responses. A–E.

A–E. RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, traces are mean of triplicate wells) for kava extract (*Mahakea* starting material) fraction induced calcium responses. Experiments were performed in 1mM CaCl₂ and nominally calcium free (~0mM CaCl₂ with 1mM EGTA) external solutions. The latter have been offset from the former by ~15 RFU in order to view traces clearly. Open bar indicates baseline period prior to stimulus addition. Black bar represents period of exposure to stimulus. Responses to vehicle (black trace), ionomycin (500 nM, red trace) and fractions (F4–8) from the batch 1 (B1) HPLC protocol (HPLC—Shimadzu Sunrise C18 column, 10 × 250 mm, 5 micron particles, eluted w/ acetonitrile:water:formic acid (400:600:1) @ 3 mL/min), are shown.

| Fraction | release | influx | SOCI | Non-SOCI |
|----------|---------|--------|------|----------|
| F4 | - | - | - | - |
| F5 | + | + | + | +/- |
| F6 | - | ++ | - | ++ |
| F7 | +++ | +++ | + | ++ |
| F8 | + | ++ | ++ | +/- |

Table 1. Summary of differential induction of release and influx response intensity induced by the indicated B1 fractions in RBL2H3.

SOCI, store-operated calcium influx (via I_{CRAC}); non-SOCI, nonstore-operated calcium influx (TRP and other channels)

Finally, we asked whether aqueous samples prepared using standardized methodology from a range of traditional Hawai'ian cultivars [1] displayed differential ability to initiate store release and influx, and SOCI versus non-SOCI. Table 2 summarizes these data sets. Root powder from the indicated that kava cultivars were prepared as described, and calcium release and influx responses were compared. Relative intensities between cultivars were calculated by normalizing to the maximal responses initiated by addition of the calcium ionophore ionomycin. These data show that the rank order of release activity by these criteria is *Kū makua* > *Papa kea* > *Papa 'ele 'ele* \approx *Mahakea* > *Hanakāpi'ai*. Rank order of influx responses over all (SOCI + non-SOCI) was *Hanakāpi'ai* > *Papa kea* \approx *Kū makua* > *Papa 'ele 'ele* > *Mahakea*. All cultivars displayed SOCI but varied strikingly in their ability to induce non-SOCI. *Hanakāpi'ai* in particular has strong non-SOCI responses.

| Root Powder | comparison | | normalized K/I | | SOCl | Non-SOCl |
|-----------------------|------------|--------|----------------|--------|---------|------------|
| | release | influx | release | influx | | |
| <i>Mahakea</i> | + | + | 0.2 | 0.2 | + (1.0) | + (1.0) |
| <i>Papa kea</i> | ++ | +++ | 0.38 | 0.69 | + (1.1) | ++ (3.0) |
| <i>Papa ‘ele ‘ele</i> | + | ++ | 0.21 | 0.55 | + (2.9) | + (1.2) |
| <i>Hanakāpi‘ai</i> | + | ++++ | 0.05 | 0.97 | +(0.5) | ++++ (6.6) |
| <i>Kū makua</i> | +++ | +++ | 0.7 | 0.65 | + (1.7) | + (1.0) |

Table 2. Comparison of calcium release and influx responses induced by 15 aqueous extracts from various Hawai‘ian ‘Awa cultivars.

RBL2H3 were loaded with Fluo-4 and assayed in bulk (50,000 cells per well, mean of triplicate wells) for kava extracts (1% aqueous solution of ground root powder for the indicated cultivars). The comparison columns summarize the relative intensity of release and influx responses induced by the 1% extracts. The normalized K/I factor expresses each cultivar-induced response as a proportion of the ionomycin-induced response (calculated from areas under the curve, $n = 3$ for each sample). Ionomycin-induced release and influx responses would be 1.0. SOCl, store-operated calcium influx (via I_{CRAC}); non-SOCl, non-store-operated calcium influx (TRP and other channels). Numbers in parentheses represent ratios of maximum response amplitude with *Mahakea* set as the unitary response.

2.5. Discussion

Kava plants are likely to contain a diverse secondary metabolome, with hundreds of compounds that can impact the physiological responses of human cells and tissues [8, 36, 43-45]. The focus of the ‘Awa field upon the kavalactones is linked to the strong likelihood that these compounds’ ligation of CNS GABA receptors is responsible for the relaxant and anxiolytic effects of the drink and its supplements [46, 47]. However, the physiological (and possibly pathophysiological) effects of kava may be underestimated by a unilateral focus upon the kavalactones. The secondary metabolome of *Cannabis sativa* provides an analogy here. For decades the primary focus of the field, the marijuana growing community and medicinal marijuana proponents has been on the major cannabinoid compounds Δ^9 -THC cannabidiol and cannabinal. These are indeed the main CNS-active components, but they and their derivatives comprise ~7 of the > 400 known bioactive molecules in *C. sativa*. Indeed, until the so-called ‘entourage’ of terpenes,

alkaloids, etc., was factored into cannabinoid pharmacology [48, 49], our understanding of its mechanisms and breadth of effect was severely limited. Similarly, the 'Awa field may now benefit from examination of the *P. methysticum* 'entourage'. Our current and previously published data suggest that effects on intracellular free calcium may provide a convenient assay system for assessment of non-kavalactone pharmacology of 'Awa.

As in our previous study, the relationship between kavalactones and calcium-mobilizing activity seems minimal [37]. LeBot assembled a comprehensive analysis of kavalactone abundance in air-dried root preparations of individual Hawai'ian 'Awa cultivars [1, 57, 64]. There is no obvious correlation between the ordering of kavalactone abundance in these cultivars and the ordering of their capacity in terms of initiating calcium responses. Notably for at least one cultivar (*Papa 'ele 'ele*), this is an inverse relationship. It should be noted that, since kavalactones are GABA-ergic and some reports suggest that GABA receptors are in mast cells and/or basophils, we examined the effect of GABA receptor inhibitors on the kava-induced non-SOCI. Inhibitors of the ionotropic and G-protein coupled GABA receptors (saclofen, bicuculline, TPMPA and CGP54626) did not affect kava-induced non-SOCI (data not shown).

A large number of non-kavalactone components have been described for *P. methysticum*, of which multiple secondary metabolites would be within the family of compounds known to regulate the TRP channels (cinnamaldehyde, cinnamic acid, capsaicin/piperidine and vanillin) [8, 36, 43, 44]. In contrast, we are reduced to speculation about likely mechanisms for the manner in which kava components might regulate calcium store depletion and SOCI. These putative mechanisms would frame further experiments, and are: (i) that the kava samples contain ligands for receptors (e.g. $G\alpha_q$ -coupled GPCR) that mobilize Ins (1,4,5) P₃ and initiate SOCI in that fashion; (ii) the kava samples contain compounds that are SERCA inhibitors. The latter is of particular interest when we note that thapsigargin itself is a natural plant product (a sesquiterpene lactone from *Thapsia garanica* L.), and that quinones and gingerol have also been shown to regulate SERCA activity [82]. The former model is also plausible, since plant odorants, endogenous lipids and cyclic peptides have variously been described to ligate GPCR. Recent descriptions of a kavalactone cannabinoid receptor (the GPCR

CB1) ligand and the overlap between the cannabinoid receptor ligands and TRP pharmacology are intriguing and worthy of further study [83, 84].

Calcium mobilizing activity is, of course, not only a convenient assay system in which to compare fractions, cultivars and extraction methods: as a fundamental second messenger, it is a critical mediator of cellular responses including growth, differentiation, motility and extensive transcriptional and functional responses. In cells of the immune system, of which RBL2H3 exemplify the mast cell/basophil type, calcium signals regulate cytokine and chemokine transcription and functional responses such as the release of immunological and inflammatory mediators [76, 85]. We have previously shown that kava extract-induced calcium signals are sufficient to induce inflammatory mediator release and the activation of calcium-dependent transcription factors in RBL2H3 [37]. The next steps in these experiments will now be to discern the relative potency of cultivars and the relative contributions of SOCI and non-SOCI to these functional responses. In close collaboration with the traditional practitioner and indigenous science communities of the Pacific, we can then relate functional responses in these, and other cell systems, to the physiological and pathophysiological effects of kava. It will also be necessary to extend these studies to other cell and tissue systems. Our somewhat narrow focus on the mast cell will need to be extended to other cell types that bear the signalling machinery that confers to responsiveness to kava components, and which may be involved in physiological and pathophysiological effects of kava.

A comprehensive understanding of 'Awa chemistry is of paramount importance. This generates two key considerations. First, the type of extraction to be characterized has important implications. There is a tension between fidelity to the traditional aqueous extractions of primarily root samples, and the need to analyze organic extracts of aerial and root powders that are the major nutraceutical forms of commercialized 'Awa.

There is good evidence that kava toxicity and efficacy are linked to extraction method. Since both traditional and commercial/organic extracts are public health issues, both need to be examined comprehensively. Close linkages with the traditional practitioner community allow indigenous knowledge to inform such studies, avoiding situations exemplified by the 2010-12 National Toxicology Program's comprehensive

examination of kava [69], which utilized only organic extraction methods that cannot necessarily be extrapolated to the daily ingestions that are prevalent in Pacific island communities. In section 2.4.2., we undertook 9 separate fractionation approaches, with data from just one of these fractionation and subfractionations presented here. This experience illustrated the challenges of a coupled fractionation-bioassay approach. Future experiments will instead focus upon a comprehensive ‘Awa metabolome, with an increased reliance on data mining approaches to provide candidate linkages between metabolome characteristics and effects on human physiology.

2.6. Acknowledgements

This work was funded by the Victoria and Bradley Geist Foundation (grant 45408), the National Institutes of Health BRIC P20MD006084, the NIH INBRE 2P20GM103466 and the NSF EPSCOR EPS-0903833 (all to HT), and the National Cancer Institute of the National Institutes of Health 5K01CA154758 (DK). The authors thank Drs. Chrystie Naeole and William Greineisen for editing of the MS. We gratefully acknowledge the support of Dr. H. Bittenbender (University of Hawai‘i), Dr. Kamana‘opono Crabbe (Office of Hawai‘ian Affairs), the Association for Hawai‘ian ‘Awa (Ed Johnston, Jerry Konanui and Helen Rogers) and Mr. Jonathan Yee. This work is respectfully dedicated to the late Henry Halenani Gomes.

CHAPTER 3:

3. INTRODUCTION

Results from Chapter 2 suggest that a diverse secondary metabolome is contributing to the physiological and pathophysiological effects of kava. One important factor that affects the production of metabolites is the plant's microbiome as well as the surrounding soil. In this chapter, we take the first look at the kava-associated and soil-associated microbiome. Soil and root samples were V4 16S sequenced to provide the first categorization of the kava microbiome as well as to compare and contrast the microbiome of the two important cultivars, *Papa'ele'ele* and *Hanakapi'ai*. Additionally, at the request of the 'Awa community, we examined the effect that traditional washing has on the root microbiome of the cultivar *Papa kea*. Currently, these findings are under review by the kava scientific and cultural community in a draft paper reproduced below:

Microbiome analysis of *Piper methysticum* and associated soil in medicinally, ritually and economically important cultivars

A. Showman, M. Speck, J.D. Baker, E. Johnston, J. Konanui, A.L. Small-Howard and H. Turner

3.1. Abstract

Kava is a soporific, anxiolytic and relaxant drink in widespread ritual and recreational use throughout the Pacific. Kava is prepared from the roots and stems of *Piper methysticum*, variously known as ‘Awa, sakau, ava, kava-kava and yaqona in Pacific Island cultures. Indigenous medicinal knowledge suggests that there are a wide variety of therapeutic uses of kava, and these extend beyond purely GABA-ergic effects that are associated with the anxiolytic effects of its kavalactones. Emerging evidence suggest that there is likely to be an extensive secondary metabolome in kava that is relevant to human exposure. In other natural product systems (e.g. *Cannabis sativa*), the deterministic effect of the plant and soil-associated microbiomes on secondary metabolome has been demonstrated. Moreover, indigenous pharmacopeia for kava describes nuances between cultivars and growth locations that suggest a potential influence of environment upon efficacy. Here, we test dual hypotheses that significant variance exists between the microbial ecology of ‘Awa cultivars with different efficacies recognized by Pacific cultural practitioners, and that traditional preparative techniques impact the ‘Awa-associated microbiome, and the degree to which ‘Awa plants influence the soil microbiome in their rhizosphere. We present the first microbial ecology study of this plant, and set the stage for future studies of the links between microbiome and secondary metabolome.

3.2 Introduction

Drinks prepared either ritually, or recreationally, from the ground rhizome and root of *Piper methysticum* play a key role in Pacific island cultures [52-56]. The beverages ‘Awa, sakau, ava, kava-kava and yaqona (Hawai‘i, Micronesia, Tonga and the Marquesas Islands, Fiji) play a role in traditional consensus building, ceremonial community events and ethnomedicine, [54, 57, 58]. Recent commercialization and nutraceutical development have also extended kava’s use to a global audience. The globalization of kava juxtaposes Western drug discovery approaches with Traditional Pharmacological Knowledge (TPK) [1, 15, 19, 59, 86]. Kava (as beverage made from

brewed root or other plant parts) is indicated in traditional Pacific medicine for urogenital conditions (gonorrhoea infections, chronic cystitis, difficulty urinating), reproductive and women's health (for menstrual problems and dysmenorrhea, to facilitate delivery, to stimulate milk production, its leaves as an abortifacient and contraceptive), gastrointestinal distress, respiratory ailments (asthma, coughs, and tuberculosis), skin diseases and topical wounds, and as an analgesic. Significant subtlety and nuance in TPK attends the precise strain, plant component and preparative method to be used [54, 55, 57-59].

As the ethnomedical understanding of kava begins to cross cultural boundaries, a rich vein of traditional wisdom that relates certain cultivars and specific growth locations/environmental conditions to specific indications and therapeutic efficacy is being exposed. This creates an opportunity for a network pharmacology approach, where the nuanced genetic and environmental influences on secondary metabolome can be elucidated and related to efficacy using modern systems biology approaches. There is also a long-term opportunity to reconcile the cultural understanding of the relationship between nuanced differences in the source material and efficacy between different growing locations, cultivars and agricultural practices, with a detailed understanding of the resultant change in secondary metabolite profile. We are approaching this process with parallel efforts in assembling a secondary metabolome and an accompanying microbial ecology profile (microbiome) for 'Awa plants. It is the latter that we report in this study.

The production of secondary metabolites is highly energy intensive and relies on a plethora of enzyme-catalyzed synthetic pathways that are not yet fully defined. These synthetic pathways are sensitive to changes in the physical and biological environment of the whole plant. Physical influencers of secondary metabolome include light (wavelength and intensity), water, salt, temperature, CO₂ and nutrient availability. These abiotic factors can dramatically influence secondary metabolite production over short timeframes. In addition, biotic factors, genetic, epigenetic and the plant's associated microbial communities, also profoundly influence the secondary metabolome. As an example, a study in *C. sativa* showed that microbiome influences THC and other key metabolite levels, and this study is reinforced by others in the literature [87-89].

Plants bear a complex leaf and stem microbiome, and specialized compartments such as flowers and nodes may exhibit even more specific microbial associations. All land plants associate with a soil microbiome, with a bidirectional influence of the host plant upon its micro-biosphere, and the soil microbiome upon plant physiology and responses. Root physiology and metabolism affects the microbiome in soil through influence on soil acidity (via root metabolism and oxidative phosphorylation) and O₂ levels. Plants also produce messenger molecules that influence microbial health and diversity (community structure) such as anti-microbial (defense) peptides and molecules that affect quorum sensing by the microbial population. Root-derived carbon, both from decomposition and secreted chemical that contain energy-rich carbon-carbon bonds, provides vital energy that again drives complexity and extent of the associated microbial population. Conversely, the microbiome is a major determinant of plant health and success. As mutualists, these microbes provide nutrients, provide resistivity towards environmental stressors (such as soil acidification, chemical stressors, water limitations) and they synthesize hormones and messengers that regulate growth and productivity.

In the current study, we take the first step towards assessment of microbial influence on secondary metabolism in 'Awa by defining the soil and plant microbiomes associated with two strains of Hawai'ian 'Awa. The microbial ecology of these plants has not previously been studied, and our data present the structure of three communities (plant, washed plant, and plant-associated soil) across two culturally significant cultivars of *Piper methysticum*, *Hanakapi'ai* and *Papa 'ele 'ele*. The influence of washing techniques in widespread use by cultural and recreational kava users upon microbial community structure is also examined in the *Papa kea* cultivar. The data suggest significant differences between cultivars (*Hanakapi'ai* and *Papa 'ele 'ele* samples) as well as significant differences in the make-up of the microbiome between washed and unwashed samples of *Papa kea*.

3.3. Materials and Methods

3.3.1 Sample collection:

Samples of 'Awa cultivars *Hanakapi'ai*, *Papa 'ele 'ele* and *Papa kea* were generously supplied by the Association for Hawai'ian 'Awa, Hilo, Hawai'i. Each three-year-old

plant sample was harvested at Pepe'ekeo, HI 96783, (approx. GPS coordinates: N 19.831551 W -155.106159) in July 2013, having been cultivated in Hawai'ian well-drained, dark-brown/reddish-brown silty clay loams (Kaiwiki series, Soil Survey of Island of Hawai'i, State of Hawai'i, Dec 1973). Voucher specimens of *Piper methysticum* are held by the Bishop Museum, Honolulu, Hawai'i. Annotated specimens and germplasm of the Hawai'ian cultivars traditionally named here are documented in the archives of the Association of Hawai'ian 'Awa, Hilo, Hawai'i [1]. The cultivar library of the Association for Hawai'ian 'Awa contains freely available specimens of the cultivars used here. Samples analyzed for the comparative cultivar microbiome were soil with roots of *Papa 'ele 'ele* and *Hanakapi'ai* in addition to bulk soil samples collected approximately one meter from the root base of the plants. Samples for the comparison of traditional washed roots vs. unwashed roots consisted of *Papa kea* roots with loose soil brushed away (unwashed) and *Papa kea* roots washed with tap water through a high power washer that results in removal of some of the epidermis and rhizosphere.

3.3.2. Sample Analysis Methods:

Root and soil samples were processed (Second Genome, South San Francisco, CA). Samples were processed in a Good Laboratory Practices (GLP) compliant laboratory running Quality Management Systems and Standard Operating Procedures (Second Genome, South San Francisco, CA) for sample and data tracking. QC and QA metrics are maintained for all sample handling, processing and storage procedures. Microbial DNA was extracted using MoBio PowerPlant and PowerSoil DNA Isolation kits respectively (MO BIO Laboratories, Inc., Carlsbad, CA). Five extractions were performed on each sample. The extracted DNA concentration ranged between 16.8 to 37.9 ng/ μ L. All samples were quantified via the Qubit® Quant-iT dsDNA Broad-Range Kit (Invitrogen, Life Technologies, Grand Island, NY) to ensure that they met minimum concentration and mass of DNA.

To enrich the sample for bacterial 16S V4 rDNA region, DNA was amplified utilizing fusion primers designed against the surrounding conserved regions, which are tailed with sequences to incorporate Illumina (San Diego, CA) flow cell adapters and indexing barcodes. Each sample was PCR amplified with two differently bar coded V4 fusion

primers. 30 samples met the post-PCR quantification minimum and were advanced for pooling and sequencing. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer®. The pooled samples containing 30 16S rRNA gene V4-region amplified and barcoded samples were loaded into the MiSeq® reagent cartridge, and then onto the instrument along with the flow cell. After cluster formation on the MiSeq instrument, the amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing.

3.3.3. Data Analysis Methods:

(a) Sequence Processing. Using QIIME [90] and custom scripts, sequences were quality filtered and demultiplexed using exact matches to the supplied DNA barcodes. Resulting sequences were then searched against the Greengenes [91] reference database of 16S sequences, clustered at 97% by uclust (closed-reference OTU picking). The longest sequence from each Operation Taxonomic Unit (OTU) thus formed was then used as the OTU representative sequence and assigned taxonomic classification via mothur's bayesian classifier, trained against the Greengenes database clustered at 98%. *(b) OTU (Operational Taxonomic Unit) Filters.* Taxa are filtered to those present in at least one of the samples (Filter-1) or to taxa significantly increased in their abundance in one category compared to the alternate categories (Filter-5). For Filter-5, ANOVA (analysis of variance) was employed to calculate p-values. Additionally, q-values were calculated using the Benjamini-Hochberg procedure to correct p-values, controlling for false discovery rates. *(c) Summarization.* We consider the abundance of each OTU. In some situations we explicitly consider only the incidence (the presence or absence) of each OTU. *(d) Sampling normalization.* We employed two approaches to account for uneven sequencing depth. In chapter section 3.4.2., we selected 209,085 sequences from each sample before calculating community-wide dissimilarity measures. This removes the bias due to sequencing depth that would otherwise affect Principal Coordinate Analysis (PCoA) plots and Adonis tests, among other analyses. In section 3.4.3. of this chapter and beyond, samples were normalized to 1 billion counts, thus the relative abundance of each OTU in this report (beginning with section 3.4.3.) represents the relative abundance in a sample per billion sequences in that sample. *(e) Sample to sample distance functions.* All

profiles are inter-compared in a pair-wise fashion to determine a dissimilarity score and store it in a distance/ dissimilarity matrix. The distance functions are chosen to allow similar biological samples to produce only small dissimilarity scores. The Weighted UniFrac [92] dissimilarity utilizes the taxon abundance differences across samples but employs a pair-wise normalization by dividing the sum of differences by the sum of all abundances. We also employ the related UniFrac measure, which considers only the presence or absence of taxa. (f) Diversity, ordination, clustering, and classification methods. Alpha Diversity Measurements used include non-parametric asymptotic estimators species-based measures, the Chao 1 Index (the number of rare OTUs found in a sample using singletons and doubletons) and ACE (Abundance-based Coverage Estimator), which compare the frequency of rare species in a sample [93]. Non-parametric quantitative species-based measurements used were the Shannon Index, which quantifies the uncertainty in the species identity of a randomly chosen individual in the sample looking at the number of individuals and the number of taxa; the Simpson Index measuring 'even-ness' of a community (strength of dominance with weight towards the abundance of the most common species and varying inversely with species diversity) [94] with variance from 0-1; where, zero represents no diversity and 1, for maximum diversity [95]; Inverse-Simpson Index equivalent to one over the probability that two randomly chosen individuals will be the same species with values starting at one and the higher the value, the greater the diversity; and Fisher Index, similar to the inverse-Simpson, but assumes that species abundance follows a log distribution with increase in sample size resulting in the collection of rare species [96]. (g) Two-dimensional ordinations and hierarchical clustering maps Samples in the form of dendrograms were created to graphically summarize the inter-sample relationships. To create dendrograms, the samples from the distance matrix are clustered hierarchically using the average-neighbor (HC-AN) method. Principal Coordinate Analysis (PCoA), a method of two-dimensional ordination plotting that is used to visualize complex relationships between samples, was performed by Second Genome. PCoA uses the dissimilarity values to position the points relative to each other. Additional two-dimensional plotting via Detrended Correspondence Analysis (DCA) was conducted in RStudio [97-99]. DCA ordination shifts samples cores along each axis allowing an average width tolerance equal to one and

equalizes species tolerances without changing sample order; minimum sample score is zero for both axes and are units of beta-diversity (units of species standard deviation) [100]. (h) *Whole microbiome significance testing*. The Adonis test is utilized for finding significant whole microbiome differences among discrete categorical or continuous variables. In this randomization/Monte Carlo permutation test, the samples are randomly reassigned to the various sample categories, and the mean normalized cross-category differences from each permutation are compared to the true cross-category differences. The fraction of permutations with greater distinction among categories (larger cross-category differences) than that observed with the non-permuted data reported as the p-value for the Adonis test.

3.3.4. Data visualization:

Heatmaps: The heatmaps were generated using the phyloseq package for the R statistical software [98], which incorporates parts of the NeatMap package for R [101]. Methods suited for calculating ecological distances and ordination positions were chosen for calculating cell values. These include the Detrended Correspondence Analysis (DCA) ordination and the weighted UniFrac distance methods, which follow the same methodology used for our ordination plots. DCA considers the underlying species distribution and may more closely follow the species gradient amongst samples. The weighted UniFrac takes into account relative species abundance as well as phylogenetic distances while calculating the distance matrix.

Circular Trees: Circular trees were used to display the phylogenetic relationships among differentially abundant OTUs. A Welch test is performed across the two groups of samples using the abundance metrics. Those OTUs significantly below the chosen p-value threshold were then grouped into families, and the one OTU with the greatest difference between the two group means from each family is selected for inclusion in the circular tree. When a family contains OTUs that are significantly increased in the second group relative to the first, and also OTUs that are significantly decreased, one OTU exhibiting each pattern is selected. A representative 16S rRNA gene from each of the resulting OTUs is then aligned and used to infer a phylogenetic tree. The tree, taxonomy labels and abundance data are rendered in iTOL [102]. The rings around the tree

comprise a heatmap, and the order of samples from innermost to outermost represents unwashed and washed roots, respectively. Heatmap Z-scores are represented by teal to red with red indicating the OTU was more abundant in that sample than in the mean of the baseline samples, and teal indicates the OTU was less abundant. The color saturation indicates the degree of difference from the mean value of the baseline samples.

3.3.5. Statistical Analysis:

Sequence processing, summarization, normalization, sample-to-sample distance metrics, ordination/clustering and significance testing were conducted using Second Genome's PhyCA-Stats™ (South San Francisco, CA) analysis software package was used for multivariate statistical analysis.

3.4 Results

3.4.1. Archaea and bacteria taxon richness at the phylum level between kava cultivars with and without pre-ingestion washing protocols.

The samples that we chose to compare were in two broad categories: (a) comparison between cultivars, (b) between soil closely associated with the root ball and a more distant bulk soil sample used as a control, and (c) within a cultivar which had been treated, or not, with a specific washing protocol in use by the indigenous community and proposed by them to be associated with improved quality of the resulting drink. Five replicates from each sample were obtained for microbiome sequencing and included Sample 1: *Hanakapi'ai* roots with soil (HS), Sample 2: *Hanakapi'ai* bulk control soil (HC) obtained approximately 1 meter from root ball, Sample 3: *Papa 'ele 'ele* root samples containing soil (PS), Sample 4: *Papa 'ele 'ele* bulk control soil (PC) obtained approximately 1 meter from root ball, Sample 5: *Papa kea* ground root/stump washed (RC), and Sample 6: *Papa kea* ground root/stump unwashed (RD). See Figure 7. for a schematic of the sampling scheme and Table 3 for a list of samples, cultivar and type or treatment where applicable. .

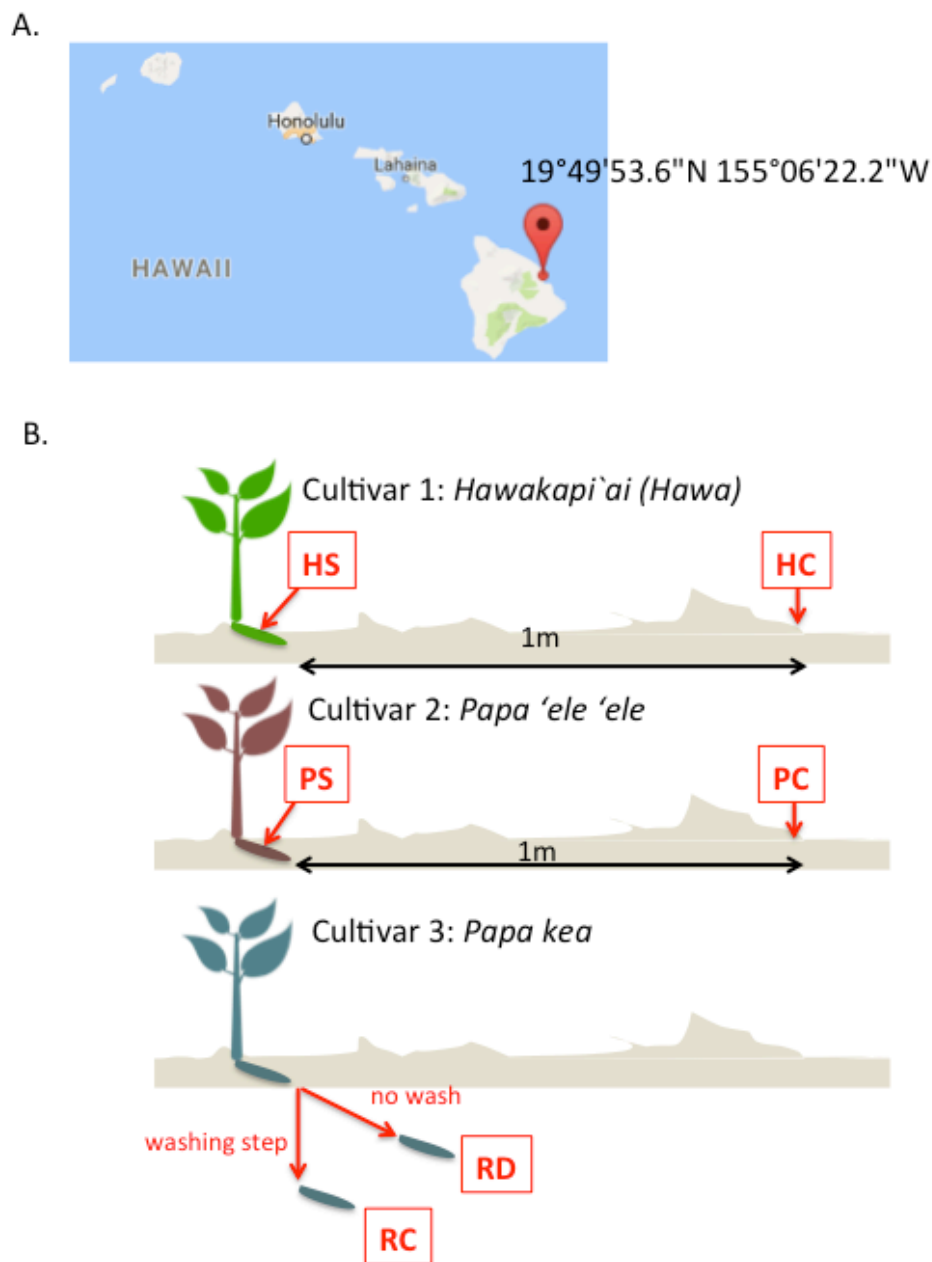


Figure 7. Cultivar Location and Sampling Schematic.

A. Samples of 'Awa cultivars *Hanakapi'ai*, *Papa 'ele 'ele* and *Papa kea* were generously supplied by the Association for Hawai'ian 'Awa, Hilo, Hawai'i. Pepe'ekeo, HI 96783 (approx. GPS coordinates: N 19.831551 W -155.106159). **B.** We obtained samples of *Papa 'ele 'ele* and *Hanakapi'ai* roots with soil and bulk soil control collected approximately one meter from the root base of the plants. Traditional washed roots vs. unwashed roots consisted of *Papa kea* roots with loose soil brushed away (unwashed) and *Papa kea* roots washed with tap water through a high power washer.

| Cultivar | Sample IDs | Sample Type |
|-----------------------|------------------------------|-------------------|
| <i>Papa kea</i> | RC.1, RC.2, RC.3, RC.4, RC.5 | Washed roots |
| | RD.1, RD.2, RD.3, RD.4, RD.5 | Unwashed roots |
| <i>Hanakapi'ai</i> | HS.1, HS.2, HS.3, HS.4, HS.5 | Roots w/ soil |
| | HC.1, HC.2, HC.3, HC.4, HC.5 | Bulk Control Soil |
| <i>Papa 'ele 'ele</i> | PS.1, PS.2, PS.3, PS.4, PS.5 | Roots w/ soil |
| | PC.1, PC.2, PC.3, PC.4, PC.5 | Bulk Control Soil |

Table 3. Sample identification, cultivar and type.

List of sample IDs by cultivar and sample type or treatment as applicable (Associated with Figure 7.B.).

Sequence coverage was variable across the sample replicates ranging from a minimum of 209,085 (RC.5) sequences to a maximum of 1,243,456 (RD.1); RD.1 most likely is a result of PCR over amplification. Most sequences were classifiable to the genus level using Bayesian classifier approach at 80% confidence. P-values were adjusted to correct for false discovery rate, and 209,085 sequences from each sample were randomly selected before calculating phylum richness as an integer. Figure 8. shows that bacterial phylum richness ranged in integers from 10 to 40, whereas archaeal phylum richness ranged from integers 0 to 1. A significant difference in bacterial richness at the phylum level was observed ($p < 0.05$) in the *Papa kea* root samples (RC/RD) compared to *Papa 'ele 'ele* (PC/PS) and *Hanakapi'ai* (HC/HS) samples by using ANOVA coupled with a Tukey HSD (honest significant difference) test.

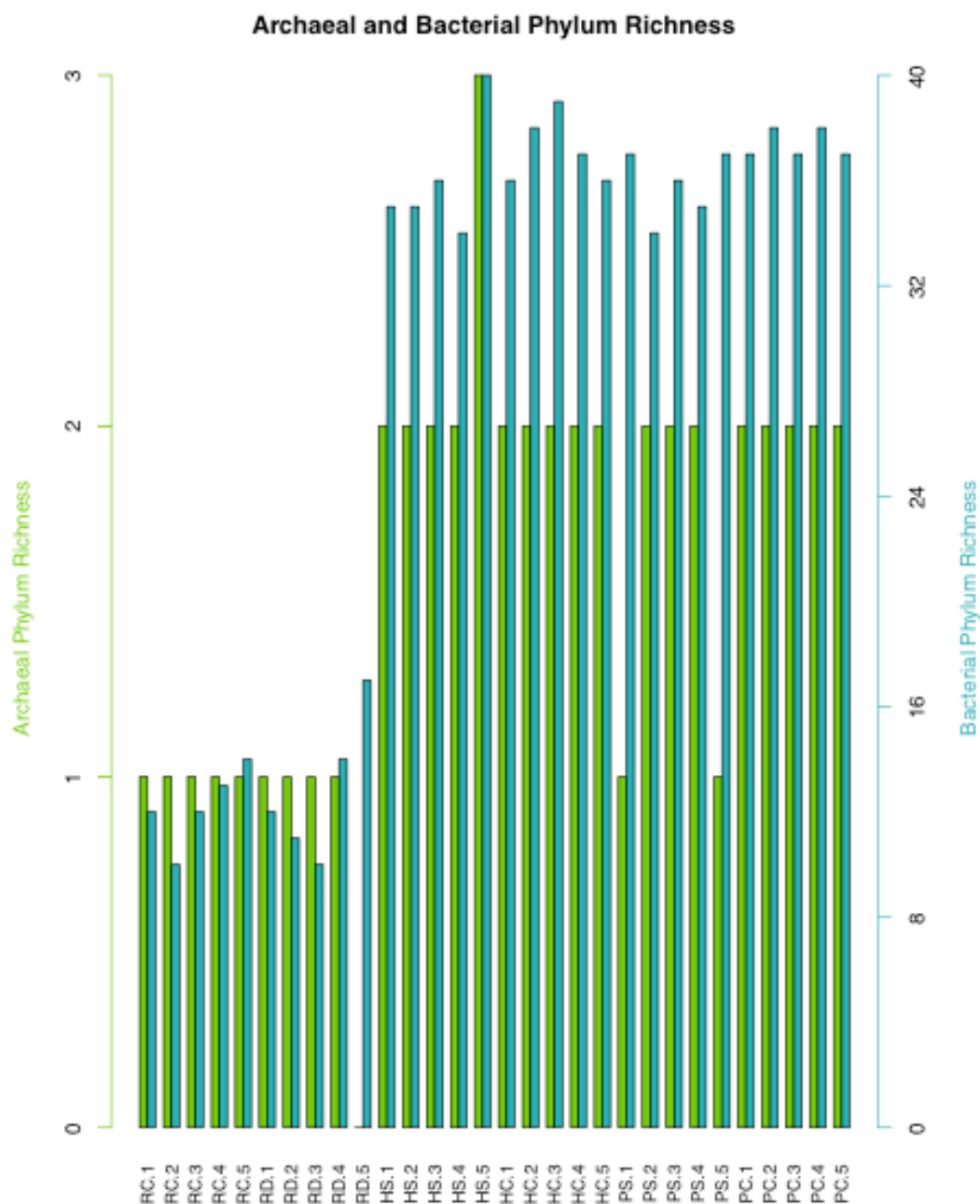


Figure 8. Archaea and bacteria taxon richness at the phylum level.

We randomly selected 209,085 sequences from each sample before calculating phylum richness. These data are displayed as Achaea (light green) and Bacterial Phylum Richness (teal). Abbreviation Identifiers are listed in Bacterial phylum richness ranged from 10 to 40, whereas archaeal phylum richness ranged from 0 to 1. A significant difference in bacterial richness at the phylum level was observed ($p < 0.05$) in the *Papa kea* root samples for both washed and unwashed compared to *Papa 'ele 'ele* and *Hanakapi'ai* soil with root and bulk soil samples by using ANOVA coupled with a Tukey HSD.

Figure 9. Phylum Composition shows the dominance of Proteobacteria (54.6% on average) was observed for all of the samples. Washed and unwashed root samples exhibit higher abundances of Proteobacteria (80.3% on average) compared to PC/PS and HC/HS samples (41.7% on average). The top 9 phyla represent on average 96.8% of each rarefied samples' sequences. For all top 9 phyla, significant differences (those with a p-value less than 0.05) in their relative proportions were detected in the RC/RD roots compared to PC/PS and HC/HS sample categories by using ANOVA coupled with a Tukey HSD. Interestingly, of the other nine phylum represented, unwashed samples showed a higher percentage of Firmicutes than PC/PS and HC/HS samples while washed samples seemed to maintain similar percentages as to these samples. Furthermore, Gemmatimonadetes were nearly undetectable in most washed and unwashed root samples of *Papa kea*.

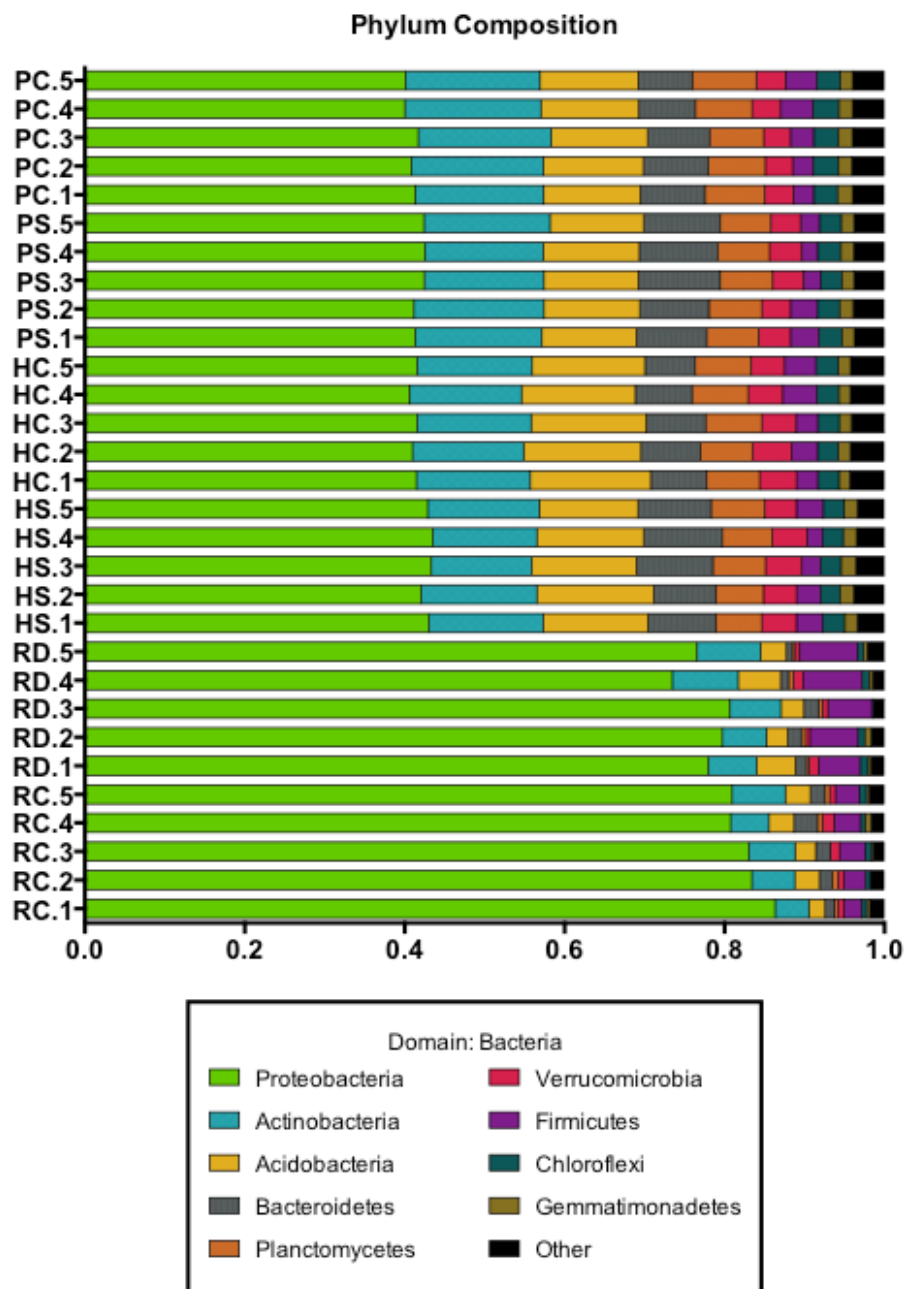


Figure 9. Phylum level composition

OTUs not classified at the phylum level were omitted from this analysis and Figure We randomly selected 209,085 sequences from each sample before calculating proportions of OTUs. These data are displayed as percent of 100 with the top nine phyla representing on average 96.% of each rarefied sample's sequence. Abbreviations are RC.1-5: washed *Papa kea* roots, RD.1-5: unwashed *Papa kea* roots, HS.1-5: *Hanakapi'ai* soil with roots, HC.1-5: *Hanakapi'ai* bulk soil, PS.1-5: *Papa 'ele 'ele* soil with roots and PC.1-5: *Papa 'ele 'ele* bulk soil. For all top 9 phyla, significant differences ($p < 0.05$) in their relative proportions were detected in the kava root sample group (washed and unwashed)

compared to *Papa 'ele 'ele* and *Hanakapi'ai* sample categories by using ANOVA coupled with a Tukey HSD.

Figure 10. shows Phylum level relative abundance across the kava root and soil samples. Random selection of 209,085 sequences shows a dominance of Proteobacteria with an average of 67.7% for all samples with washed and unwashed roots exhibiting even higher abundances of Proteobacteria with an average of 98.2% compared to PC/PS and HC/HS (average of 42.5%). The top 9 phyla represent an average of 98.2% of each of the sequences of the rarefied samples. In all top 9 phyla significant differences ($p < 0.05$) in relative abundance were detected in the washed and unwashed root samples compared to the PC/PS and HC/HS samples by using ANOVA coupled with a Tukey HSD.

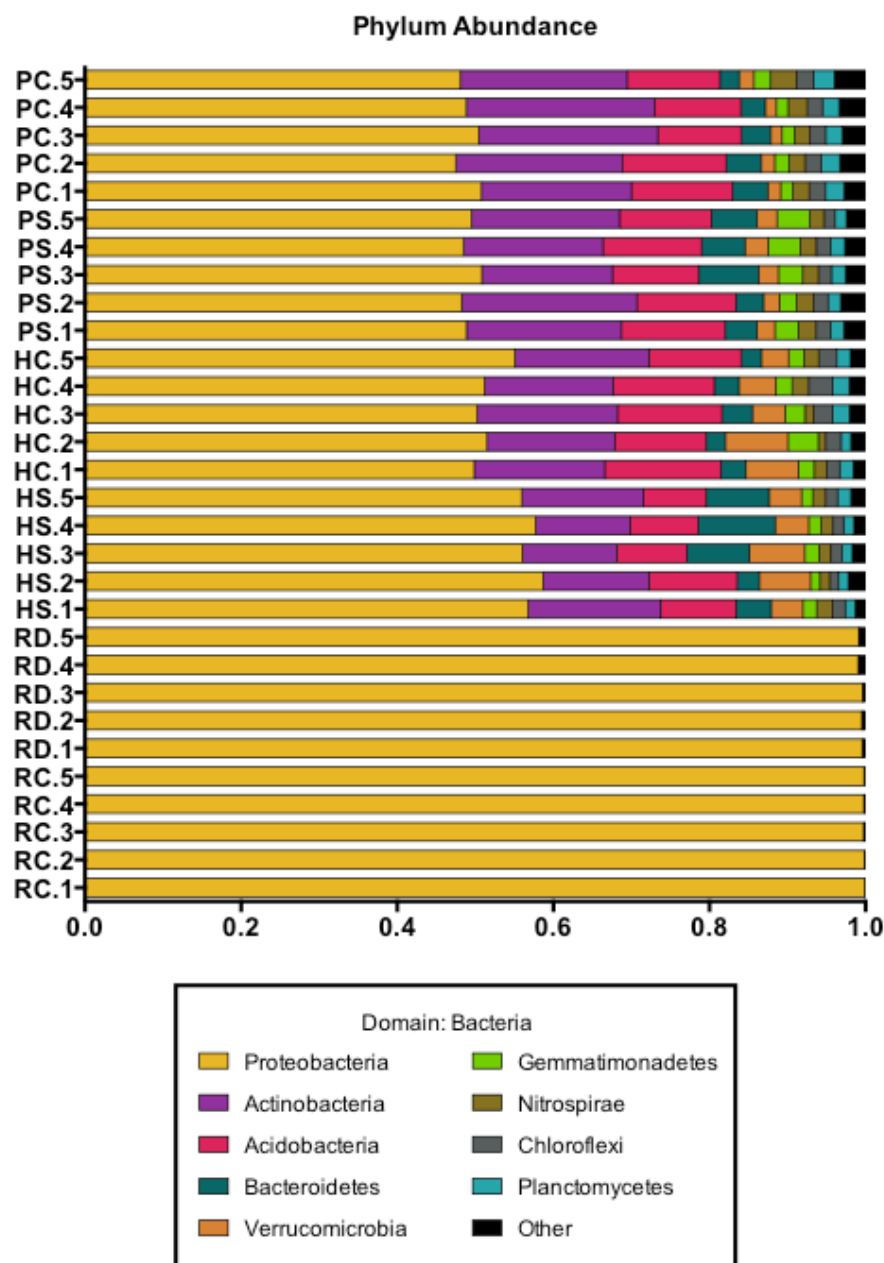


Figure 10. Comparison of phylum-level proportional abundance across samples.

The bar chart displays the 9 phyla with the largest relative abundance found by summing the sequence abundance from the OTUs within the phylum. We randomly selected 209,085 sequences from each sample before calculating phylum abundance. The top 9 phyla represent on average 98.2% of each rarefied samples' sequences. Abbreviations are RC.1-5: washed *Papa kea* roots, RD.1-5: unwashed *Papa kea* roots, HS.1-5: *Hanakapi'ai* soil with roots, HC.1-5: *Hanakapi'ai* bulk soil, PS.1-5: *Papa 'ele 'ele* soil with roots and PC.1-5: *Papa 'ele 'ele* bulk soil. In all top 9 phyla significant differences ($p < 0.05$) in relative abundance were detected in the *Papa kea* root samples group (washed and unwashed) compared to *Papa 'ele 'ele* and *Hanakapi'ai* sample categories by using ANOVA coupled with a Tukey HSD.

3.4.2. Whole Microbiome Analysis

Alpha and Beta diversity analysis (Filter 1: abundance of 13,333 taxa present in at least one sample) was considered with comparisons between between *Hanakapi'ai* HC/HS and *Papa 'ele 'ele* PC/PS samples compared to *Papa kea* washed and unwashed roots as well as individual comparisons of these treatments. Figure 11. shows that both qualitative and quantitative indices of alpha diversity generally indicate significant separation between *Hanakapi'ai* HC/HS and *Papa 'ele 'ele* PC/PS samples compared to *Papa kea* washed and unwashed roots. Simpson Index results showed moderate separation between washed and unwashed roots compared to the HC/HS and PC/PS; Inverse Simpson showed relatively significant separation of most HC/HS and PC/PS samples with only moderate separation of samples HC.2 and HS.2 versus *Papa kea* washed and unwashed roots.

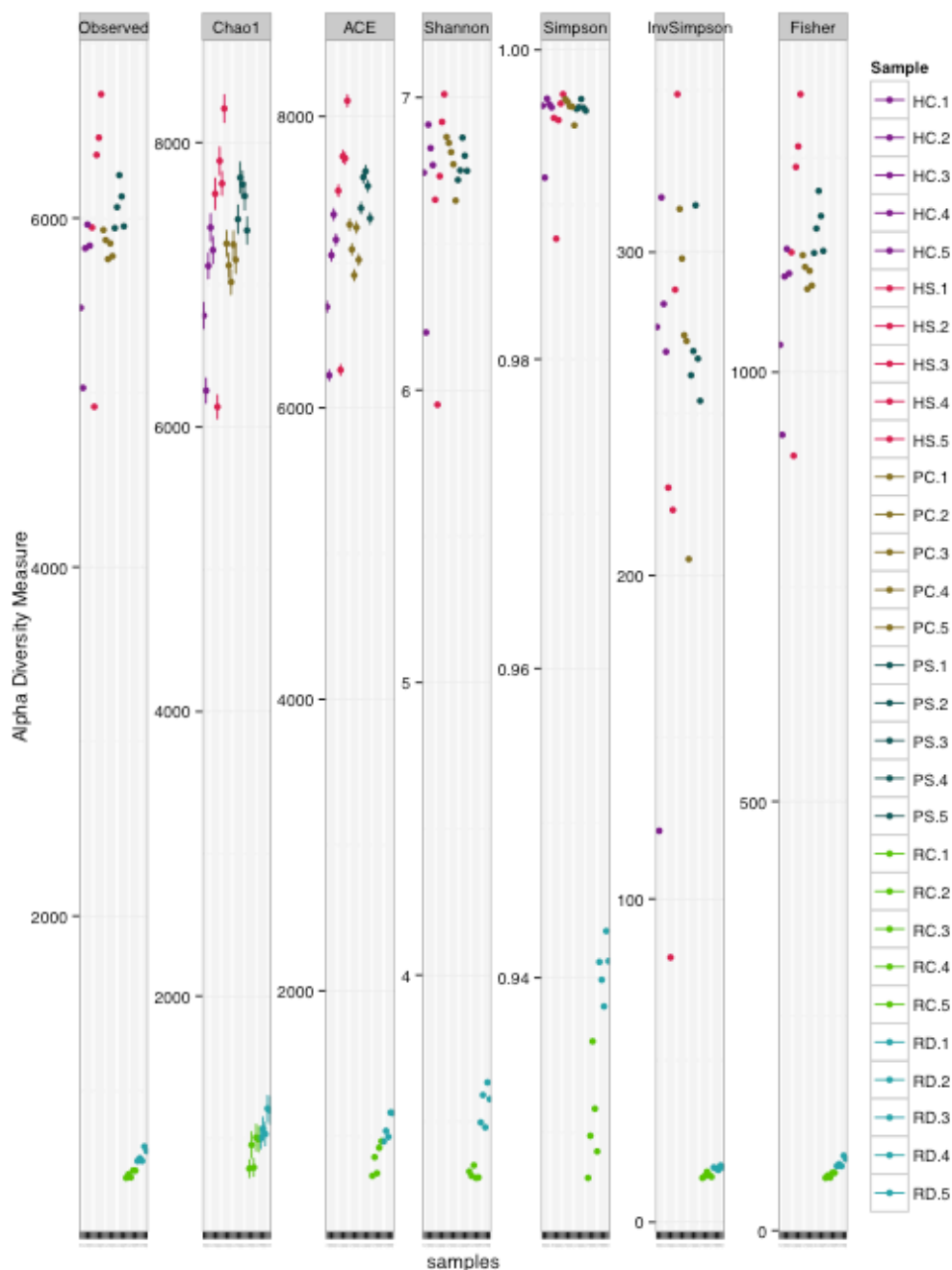


Figure 11. Ordination by sample treatment weighted.

We considered alpha diversity expressed as species richness considering data from the whole microbiome (Filter 1: 13,333 taxa present in at least one of the samples). Non-parametric, qualitative indices used: Chao1 and ACE; Non-parametric, quantitative indices used: Shannon, Simpson, Inverse Simpson and Fisher. Horizontal axes from left to right are in order of Sample key from top to bottom for each indices. Abbreviations are RC.1-5: washed *Papa kea* roots, RD.1-5: unwashed *Papa kea* roots, HS.1-5: *Hanakapi 'ai* soil with roots, HC.1-5: *Hanakapi 'ai* bulk soil, PS.1-5: *Papa 'ele 'ele* soil with roots and PC.1-5: *Papa 'ele 'ele* bulk soil.

| Category | Bins | Sample Count | WUnifrac | UniFrac | HC-AN Distinct | | | Differentiating OTU Count |
|-----------------------|---------------------|-----------------|----------|---------|----------------|-----|-----|---------------------------|
| | | | | | at1 | bt1 | at5 | at5 |
| Wash | uw w | 5 5 | 0.044 | 0.012 | No | No | NA | 256 |
| Roots | abs pres | 10 10 | 0.017 | 0.032 | No | No | NA | NA |
| Roots4way | HC HS PC PS | 5 5 5 5 | 0.001 | 0.001 | No | Yes | NA | NA |
| <i>Hanakapi</i> 'ai | HC HS | 5 5 | 0.009 | 0.008 | NA | Yes | NA | 1895 |
| <i>Papa</i> 'ele 'ele | PC PS | 5 5 | 0.013 | 0.004 | Yes | Yes | NA | 2796 |
| Wash_Roots | abs pres uw w | 10 10 5 5 | 0.001 | 0.001 | NA | NA | NA | NA |
| gDNAlabCon | continuous | 30 | 0.265 | 0.209 | NA | NA | NA | NA |
| Mass | continuous | 30 | 0.001 | 0.001 | NA | NA | NA | NA |
| PCRYield | continuous | 30 | 0.002 | 0.002 | NA | NA | NA | NA |
| nSeq16S | continuous | 30 | 0.409 | 0.346 | NA | NA | NA | NA |

Table 4. Microbiome differences by categories.

A significant microbiome difference was observed in samples grouped by the categories Wash, Roots, Roots4way, *Hanakapi* 'ai, *Papa* 'ele 'ele, Mass, Wash_Roots and PCRYield based on Weighted UniFrac metric and UniFrac metric on the presence/absence of 13,333 taxa. The detected microbial community relationship showed no significant dependences on gDNAlabCon and nSeq16S based on Weighted UniFrac metric and UniFrac metric on the presence/absence of 13,333 taxa.

The analysis of the impact of washing techniques employed during kava preparation was an important interest of the collaborating traditional practitioners, so additional Detrended Correspondence Analysis (DCA) of washed and unwashed samples was conducted, (Figure 12.). This indicated a variance of 89.9% (Axis 1: 77.3% and Axis 2: 12.6%), which suggests that unwashed and washed samples share few species with each other. Weighted and Unweighted UniFrac also showed a moderate separation of washed and unwashed roots with the Adonis test (metric based on 13,333 taxa present in at least one sample) yielding a p-value of 0.044 and 0.012, respectively as indicated in Table 5.

Principal Coordinate Analysis PCoA of PC/PS versus HC/HS samples (roots4way) for both Weighted and Unweighted UniFrac demonstrated a 4% and 6%

variation, respectively (data not shown). Weighted PCoA results comparing HC and PC bulk soil samples did yield an Adonis test p-value of 0.017 suggesting significant microbiome difference observed for at least one sample; moreover, a separation of microbiome between HC/HS and PC/PS (roots4way) Adonis test yielded a p-value of 0.001 along axis 2 (Table 5) indicating a significant microbiome difference was observed for at least one sample category. Weighted PCoA analysis did not reveal separation between bulk soil samples HC and PC versus roots samples HS and PS; however the Adonis test yielded a p-value of 0.032 indicating significant microbiome difference for at least one sample category. Further Detrended Correspondence Analysis (DCA) of *Hanakapi'ai* samples and *Papa* samples showed significant variance between HC and PC samples versus HS and PS samples. Figure 13. indicated a 70.9% variance between HC versus HS (Axis 1: 42.6% and Axis 2: 28.3%). Similarly, DCA analysis of *Papa 'ele 'ele* samples (Figure 14.) demonstrated an 86.1% variance between PC versus PS (Axis 1 72.1% and Axis 2: 14%). Both PCoA and DCA indicate significant differences in the microbiome of bulk soil samples and roots. Figure 15. shows Hierarchical Clustering based on Weighted UniFrac distance between samples revealed separate clusters of PC and PS samples of the factors roots4way and *Papa* while no clustering was noted for the other factors.

In summary, significant microbiome differences were observed in samples grouped by the categories Wash, Roots, Roots4way, *Hanakapi'ai*, *Papa 'ele 'ele*, Mass, Wash_Roots and PCR yield based on Weighted UniFrac metric and presence/absence UniFrac metric of 13,333 taxa (Table 5).

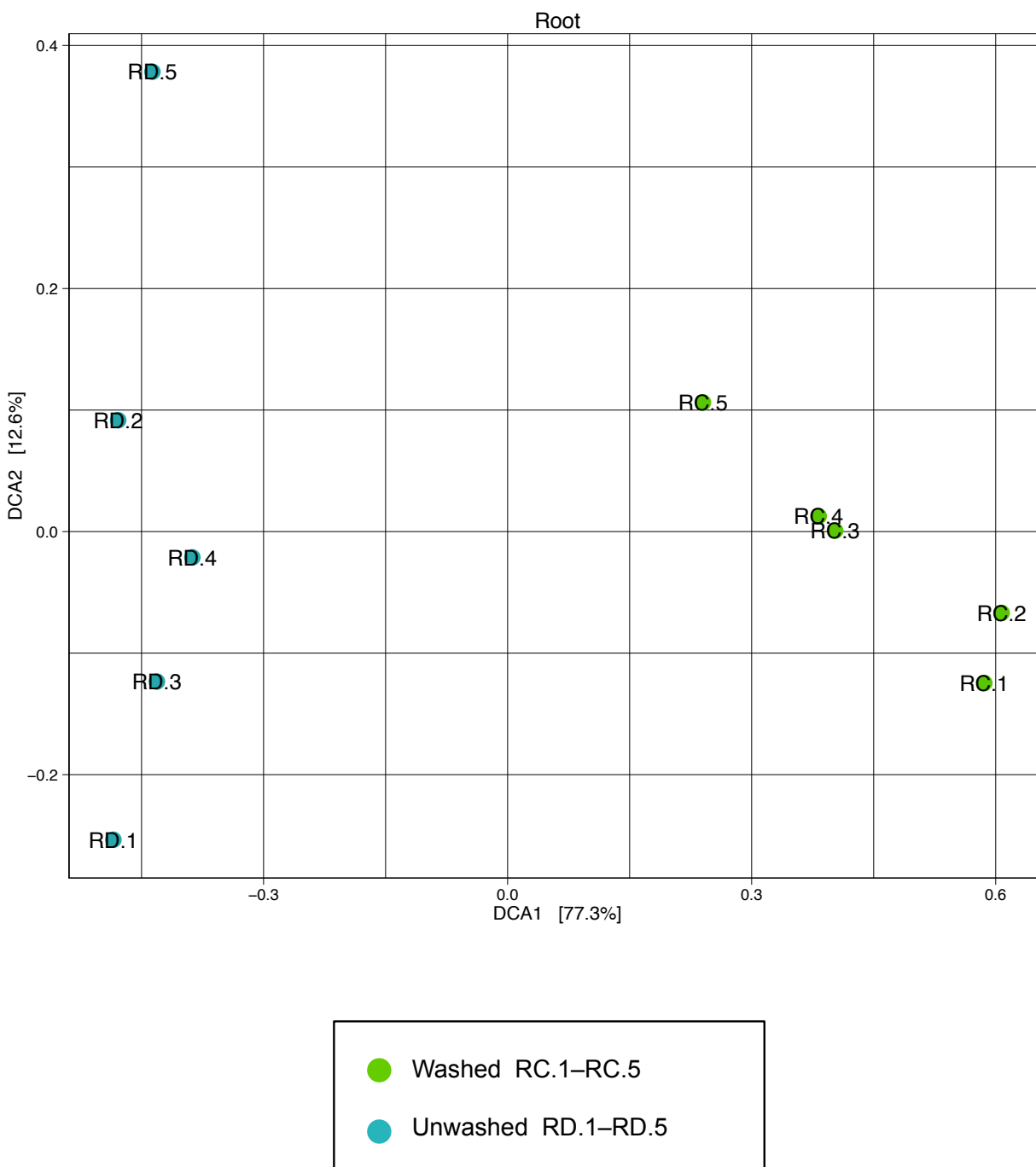


Figure 12. DCA of treatment washed vs. unwashed *Papa kea*.

Detrended Correspondence Analysis (DCA) based on weighted UniFrac distance between 1616 taxa present in at least one sample. Axes are scaled in SD units with unwashed samples on the left sharing few species with washed samples on the right.

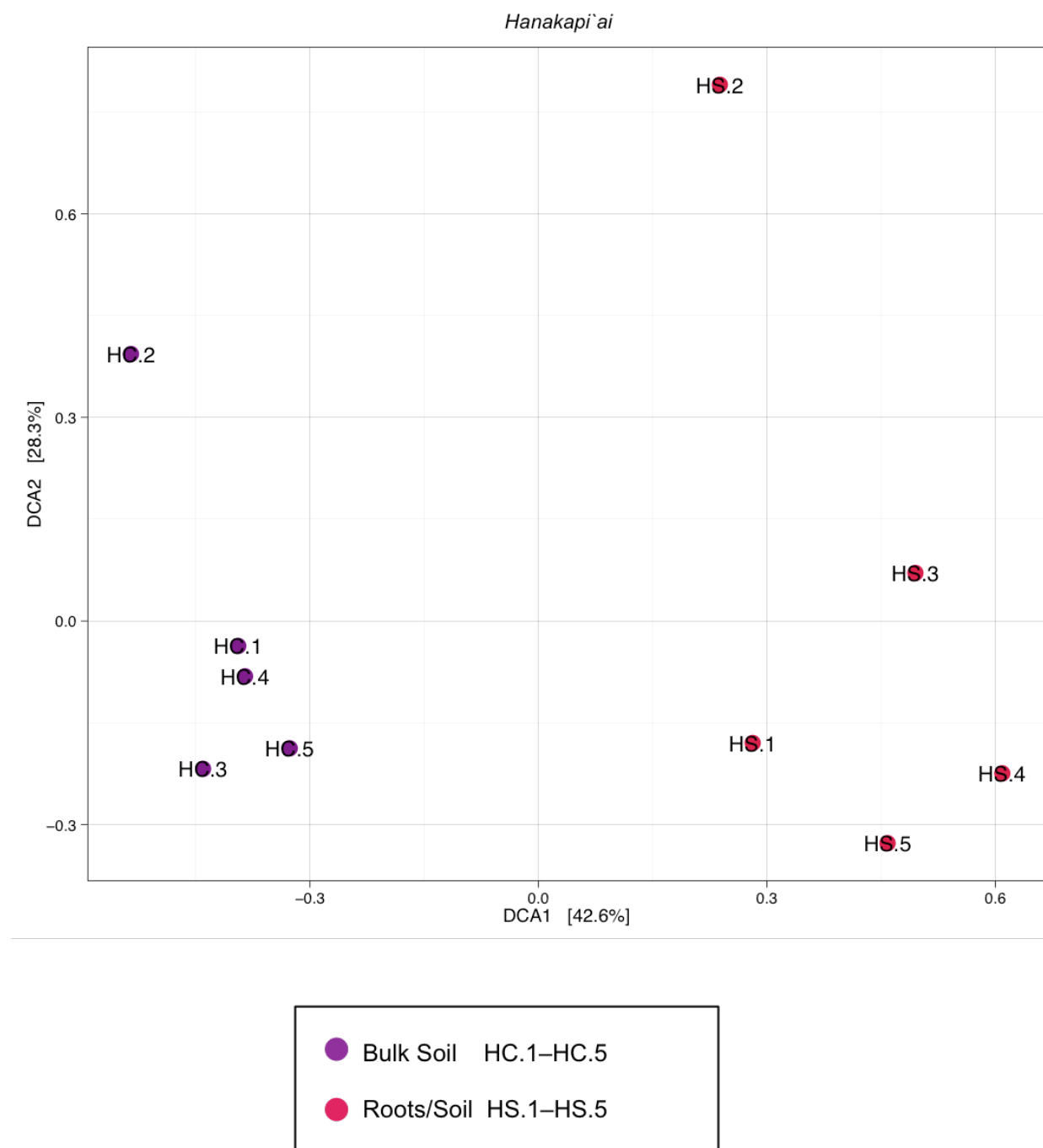


Figure 13. DCA of *Hanakapi'ai* soil with roots vs. bulk soil.

Detrended Correspondence Analysis (DCA) based on weighted UniFrac distance between 13,333 taxa present in at least one sample. Axes are scaled in SD units with bulk control soil samples on the left sharing few species with root with soil samples on the right. DCA analysis explains 70.9% of variance between *Hanakapi'ai* bulk soil and *Hanakapi'ai* soil with roots (Axis 1: 42.6% of variance; Axis 2: 28.3% of variance).

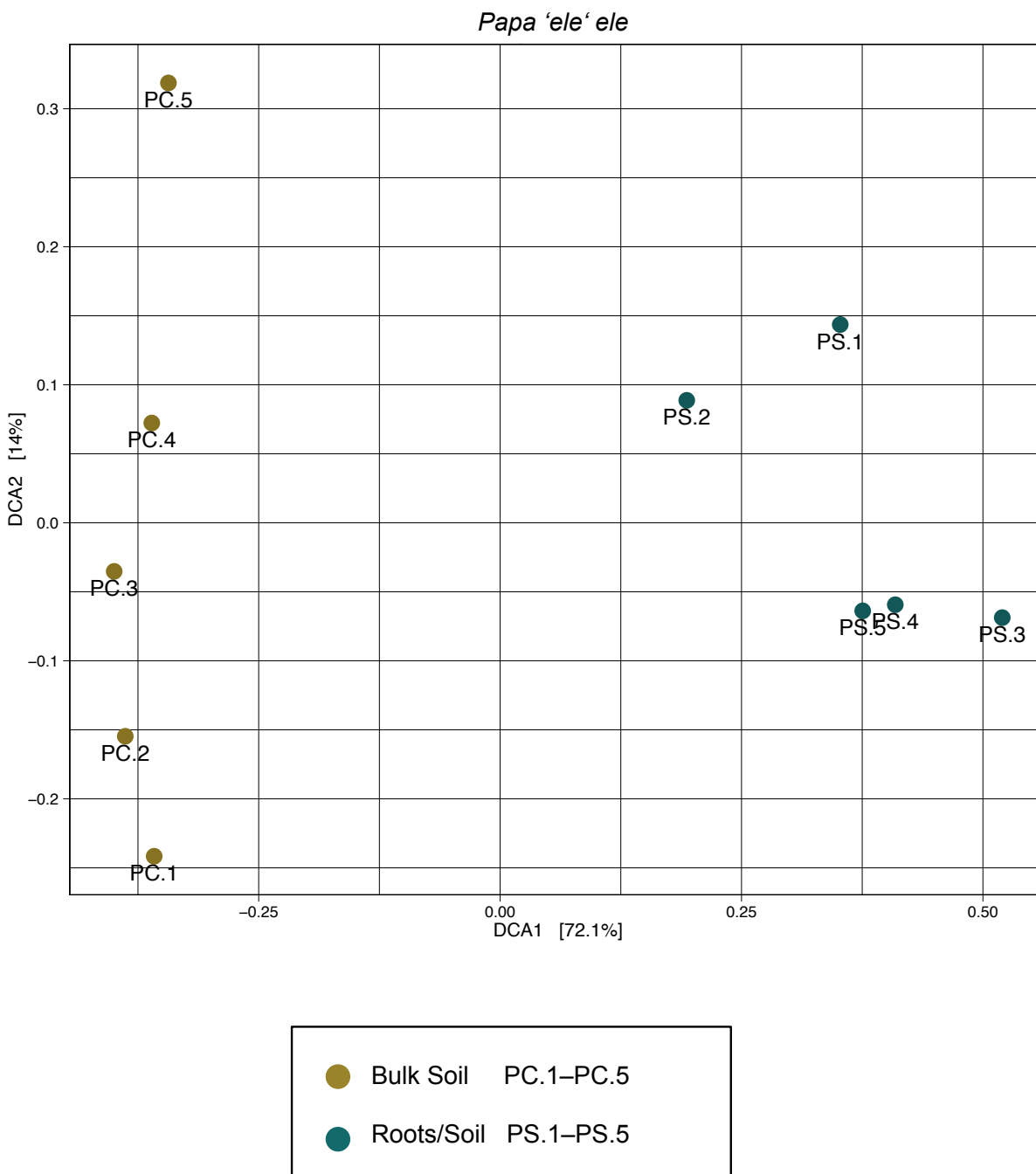


Figure 14. DCA of *Papa 'ele 'ele* soil with roots vs. bulk soil.

Detrended Correspondence Analysis (DCA) based on weighted UniFrac distance between 13,333 taxa present in at least one sample. Axes are scaled in SD units with bulk control soil samples on the left sharing few species with root with soil samples on the right. DCA analysis explains 86.1% of variance between *Papa 'ele 'ele* bulk soil and *Papa 'ele 'ele* soil with roots (Axis 1: 72.1% of variance; Axis 2: 14% of variance).

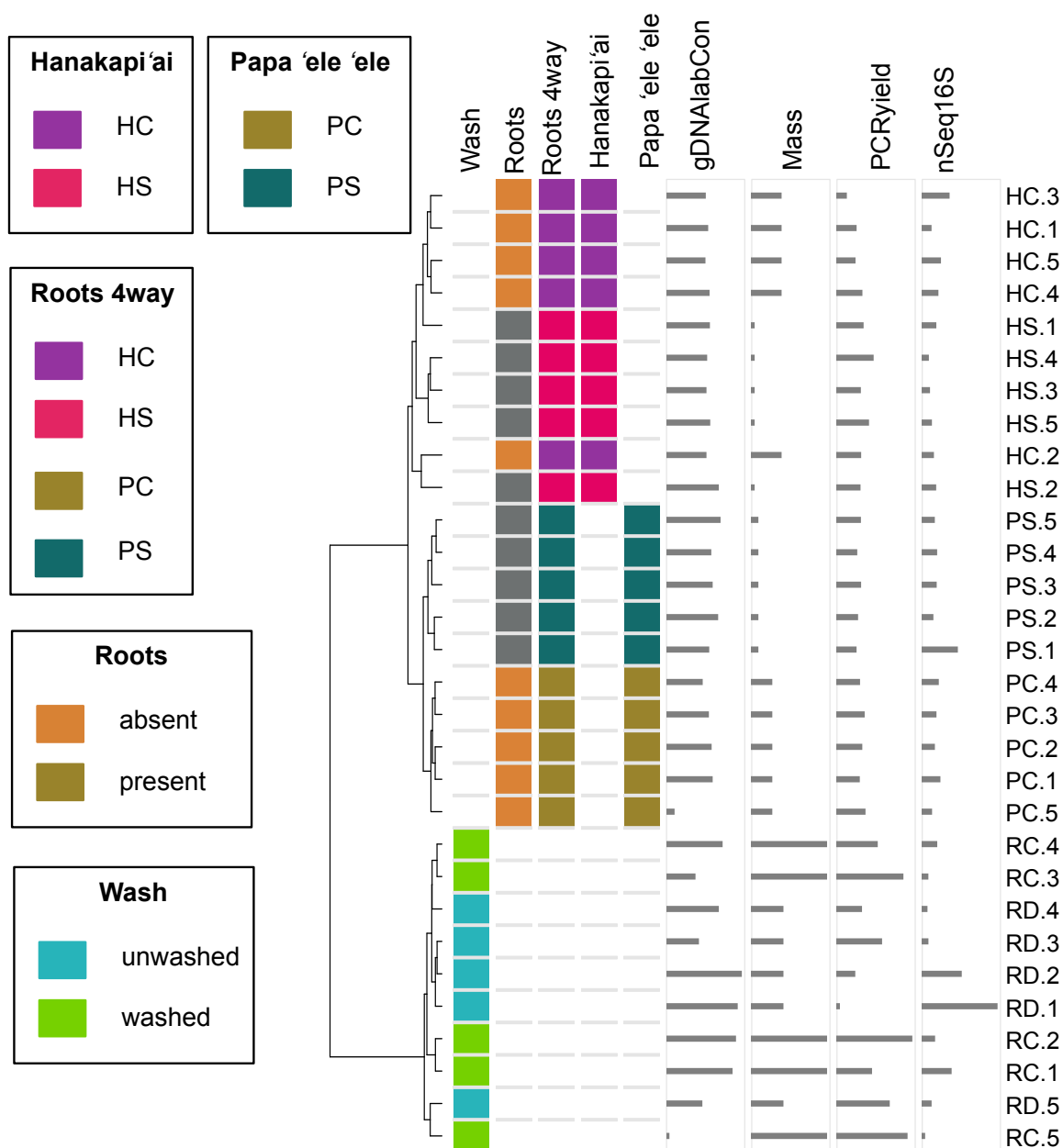


Figure 15. Hierarchical clustering weighted (average linkage).

Based on Weighted UniFrac distance between samples given abundance of 13,333 taxa present in at least one sample. HC-AN analysis based on the abundance of 13,333 taxa revealed separate clusters comprised of PC and PS samples of the factors Roots4way and *Papa 'ele 'ele*. No clusters of samples in the factors Wash, Roots and *Hanakapi'ai* were observed.

3.4.3. Washing separates OTUs

We assessed the Operational Taxonomic Unit (OTU) diversity in washed and unwashed *Papa kea* samples. Alpha and Beta diversity analysis, based on weighted UniFrac distance between 256 taxa present in at least one sample (Filter 5: taxa with significant abundance differences in one category compared to alternate categories using ANOVA) was considered with comparisons between RC (washed) and RD (unwashed) *Papa kea* root samples. Figure 16. demonstrates that both qualitative and quantitative indices of alpha diversity generally indicate moderate separation between washed and unwashed *Papa kea* samples. Specifically, Simpson and Inverse Simpson Indices showed moderate separation between RC and RD samples with replicates RC.3 and RD.2 only slightly separated; however RD.2 showed relatively high OTU diversity and largest sequence coverage (~700,000) behind sample RD.1 (1,243,456). This variance in OTUs for these two replicates may help to explain their general separation from the other unwashed replicates. Detrended Correspondence Analysis (DCA) of washed and unwashed samples as (Figure 17.) explains a variance of 84.8% (Axis 1: 75.8% and Axis 2: 9%), demonstrating differences in OTU selection due to root treatment. ANOVA Hierarchical Clustering based on Weighted UniFrac of 256 taxa (Figure 18.) revealed separate clustering of RC and RD samples based on treatment.

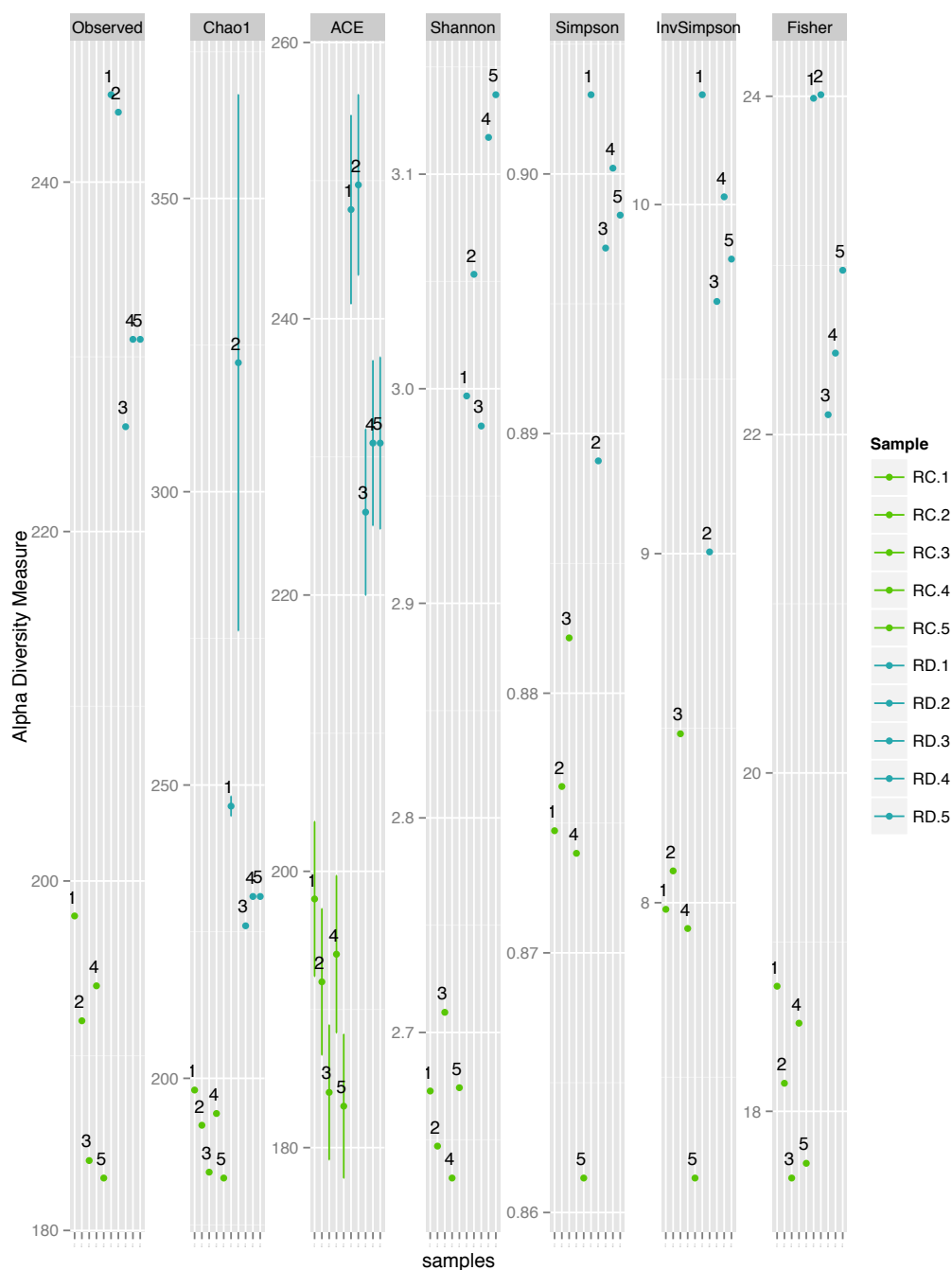


Figure 16. Ordination of *Papa kea* washed vs. unwashed.

We considered alpha diversity expressed as taxa with significant abundance (Filter 5: abundance of 256 taxa with significant abundance differences across at least one of the categories). Non-parametric, qualitative indices used: Chao1 and ACE; Non-parametric, quantitative indices used: Shannon, Simpson, Inverse Simpson and Fisher. Horizontal axes from left to right are RC.1, RC.2, RC.3, RC.4, RC.5, RD.1, RD.2, RD.3, RD.4, and RD.5 for each indices. Abbreviations are RC.1-5: washed *Papa kea* roots and RD.1-5: unwashed *Papa kea* roots.

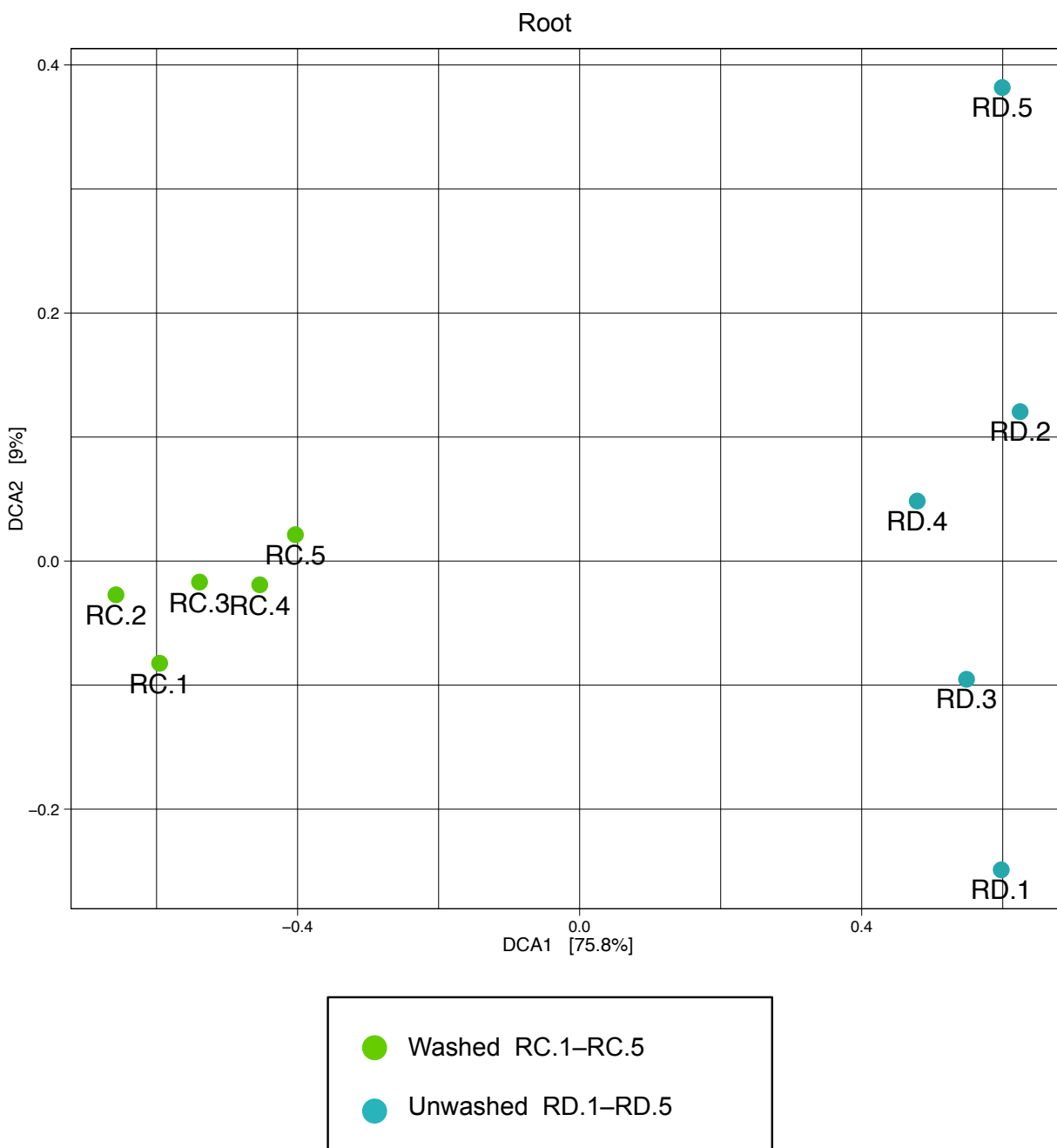


Figure 17. DCA of *Papa kea* washed vs. unwashed.

Detrended Correspondence Analysis (DCA) based on weighted UniFrac distance based on Weighted UniFrac distance between samples given abundance of 256 taxa with significant abundance differences across at least one of the categories.

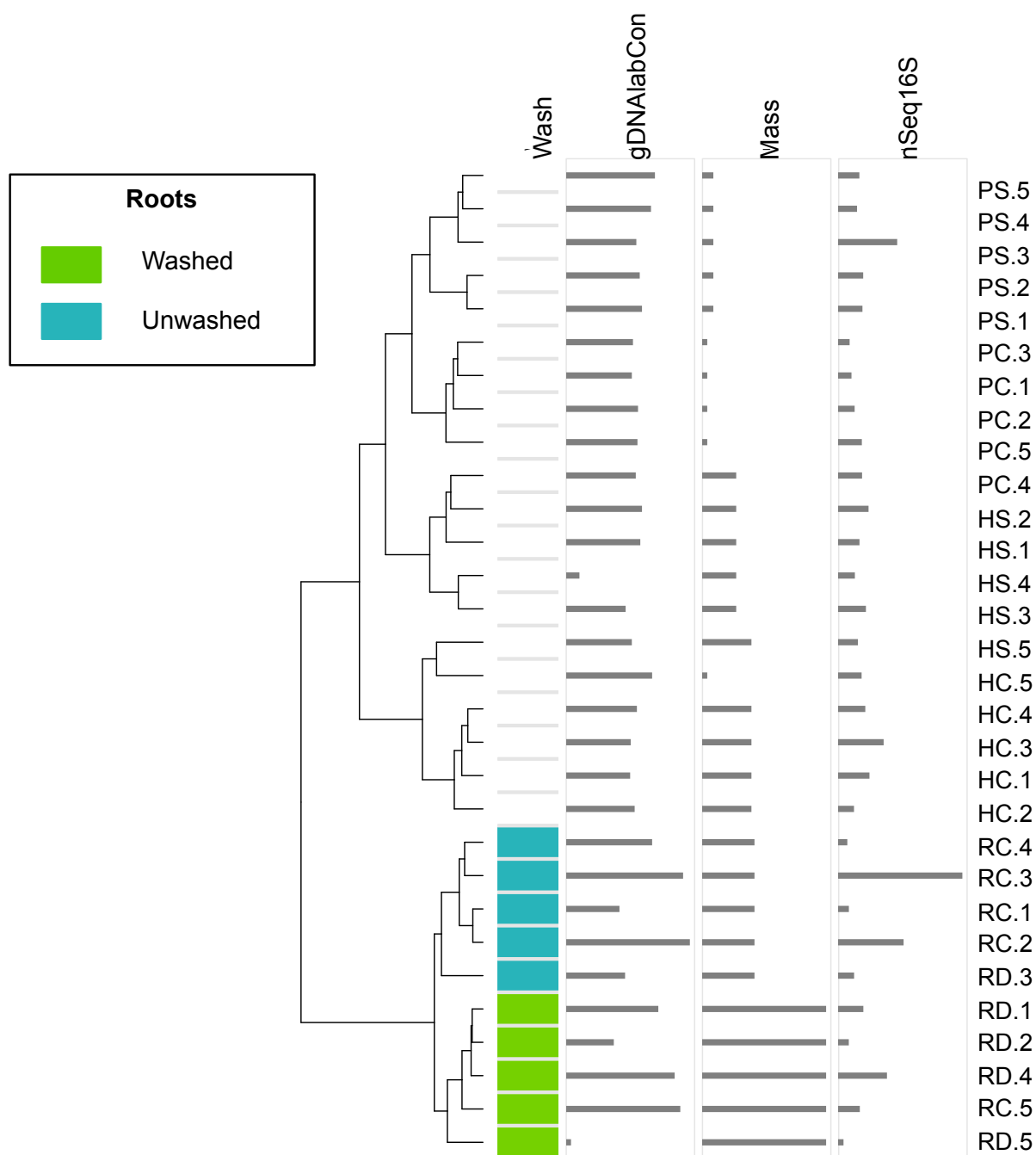


Figure 18. ANOVA hierarchical clustering weighted.

Based on Weighted UniFrac distance between samples given abundance of 256 taxa with significant abundance differences across at least one of the categories.

We investigated profiles of OTUs (Filter 5) of washed and unwashed samples *Papa kea* root/associated soil samples based on the lowest p-values. Of particular interest in these analyses are any gains in OTU diversity associated with washing as these may reflect (1) post-washing opportunistic colonization, (2) contamination from human contact during the washing process, and (3) may provide a framework for understanding practitioner assertions as to the benefits of washing. Figure 19. (with identifications of OTUs on Table 5) showed that all selected OTUs belonged to one of the top 2 phyla: Proteobacteria (11), Firmicutes (1). We asked of the 12 selected OTUs, seven showed significant increases in unwashed samples compared to washed samples including OTU 336164 (Class: Bacilli), which contains several known pathogenic species, which include *Bacillus anthracis* Cohn, *Bacillus cereus* Frankland and Frankland, and to a lesser extent *Bacillus subtilis* Cohn, *Bacillus licheniformis* Chester are all capable of causing intestinal infections usually from handling infected material or ingesting infected meat [103]. Four taxa (950606, 53498, 9939, 62346) identified to family Enterobacteriaceae, demonstrated greater selection in washed samples versus unwashed samples; many species in this family are probiotic and may support assertions of gastro-intestinal benefits from practitioners. Additionally, taxa 121180, genus *Erwinia* was also selected for in washed samples with this genus being linked to several plant pathogenic species responsible for blight on Rosaceae crops. It is not surprising that this genus would be found among the roots of 'Awa as blight has been a problem for some growers appearing on stems and as 'shot holes' in leaves and can result in plant die-back [104].

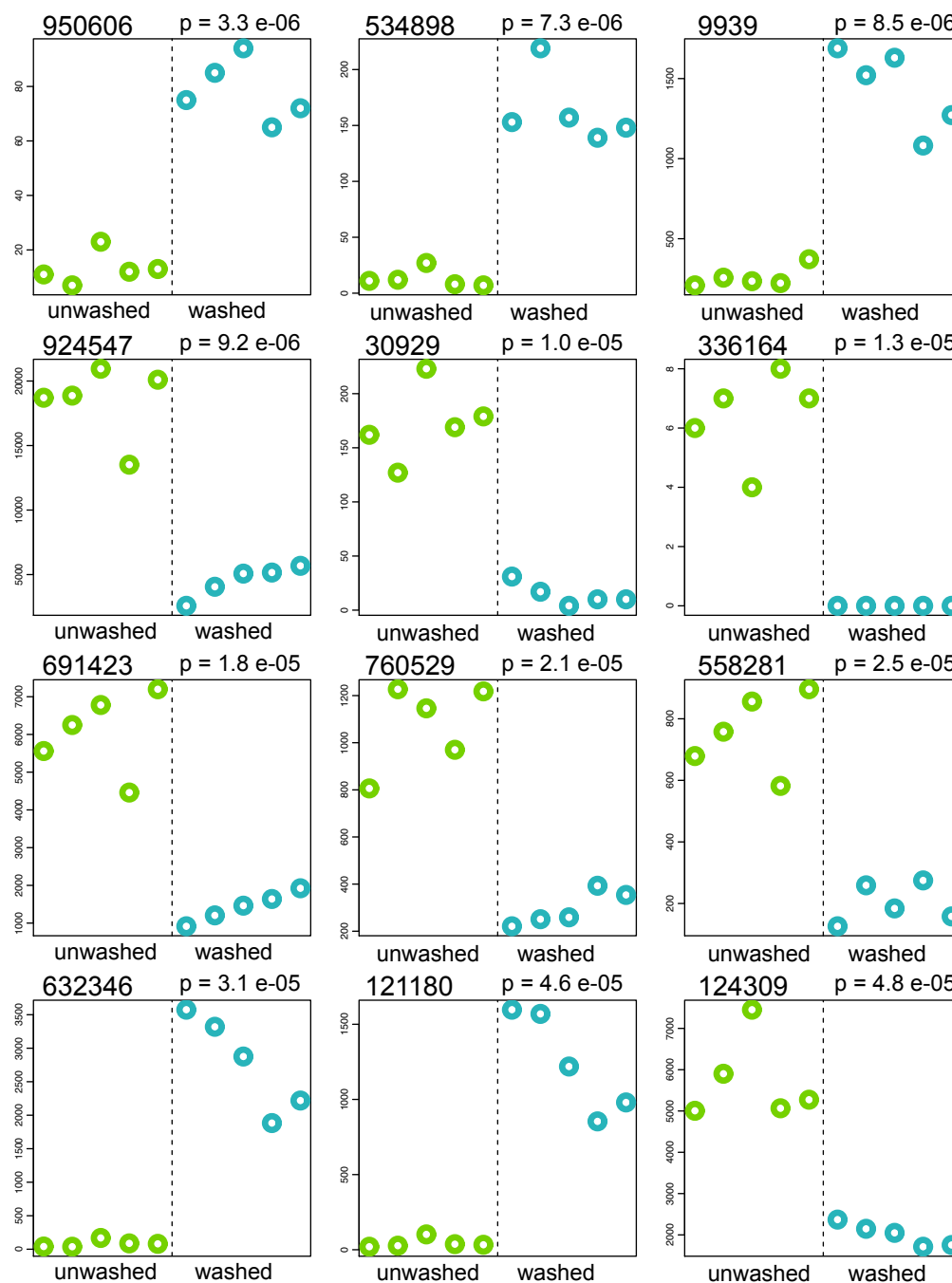


Figure 19. OTUs profiles with lowest p-values for washed and unwashed *Papa kea* samples.

Profiles of at5 OTUs generating the lowest p-values; p-values shown at top right of each OTU plot are unadjusted for multiple testing. The y-axis represents the OTU abundance. Samples are grouped and colored by category along the x-axis in the following order: RD.1, RD.2, RD.3, RD.4, RD.5, RC.1, RC.2, RC.3, RC.4 and RC.5. OTU (as listed on top left of each plot) are identified in Table 5.

| | OTU | Phylum | Class | Order | Family | Genus |
|--------|--------|----------------|---------------------|-------------------|--------------------|----------------|
| Left | 950606 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| | 924547 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| | 691423 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| | 632346 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| Middle | 534898 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| | 30929 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| | 760529 | Proteobacteria | Gammaproteobacteria | unclassified | unclassified | unclassified |
| | 121180 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | <i>Erwinia</i> |
| Right | 9939 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| | 336164 | Firmicutes | Bacilli | unclassified | unclassified | unclassified |
| | 558281 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |
| | 124309 | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | unclassified |

Table 5. ANOVA annotations of the OTUs with the lowest p-value.

Taxa identification by OTUs of the top 12 phyla (associated with Figure 19). OTUs are listed from the left, middle and right, then top to bottom as depicted in Figure No OTUs were identified to species.

Six families contained OTUs of either higher or lower abundances between washed (RC) and unwashed (RD) *Papa kea* samples, and these were compared in a heatmap tree (Figure 20.). From the 13,333 OTUs present in the study, 256 OTUs (within 25 families) were significantly different ($p < 0.05$) in one of the comparison groups. The one OTU with the greatest difference between the two group means (washed vs. unwashed groups) from each family was selected. Actinobacteria and Acidobacteria generally were less abundant in unwashed samples compared to washed samples while Firmicutes and Proteobacteria showed mixed results with Proteobacteria OTUs 108916, 950606, 926370, 219439, and 1120966 and Firmicutes OTU 839282 being higher in abundance for washed samples. The single OTU for Chloroflexi demonstrated higher abundance in washed samples; possibly reflecting extraction of these commonly plant associated bacteria from the root or opportunistic colonization due to the presence of chlorine in the wash water as many of the Chloroflexi function as anaerobic, obligate organohalide respirers converting compounds such as inorganic chlorine in to organochlorine compounds through chloroperoxidase activity [105].

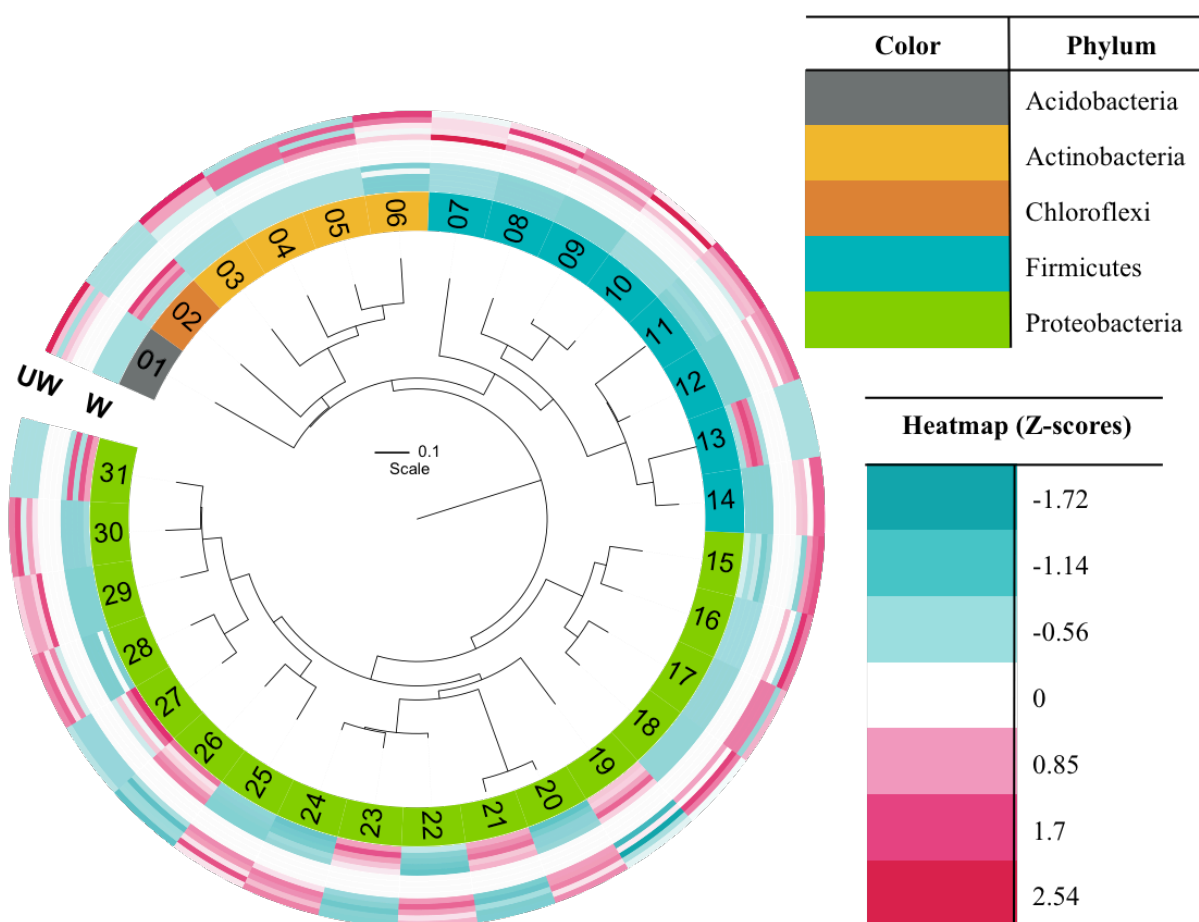


Figure 20. Heatmap tree comparing phylum of washed samples (inner rings) and unwashed samples (outer rings).

From the 13,333 OTUs present in the study, 256 OTUs (within 25 families) were significantly different ($p < 0.05$) in one of the comparison groups. The one OTU with the greatest difference between the two group means (washed vs unwashed groups) from each family was selected. 6 families contained OTUs with both higher and lower abundance scores in the washed compared to the unwashed samples. In these 6 families, both OTUs were selected. An idealized tree is computed using taxonomic classifications. The color saturation indicates z-score, which represents the OTU differences from the overall mean abundance of the OTU across samples. The z-score of one OTU in one sample is defined as the difference between OTU abundance and mean abundance of the OTU across all samples, divided by the standard variation of the OTU's abundance across all samples. Dark blue indicates the OTU has low abundance relatively to other samples (z-score of -1.72); white indicates no difference from the mean; dark red indicates high abundance (z-score of 2.54). Of the OTUs selected, those within Actinobacteria and Acidobacteria generally tended to be less abundant in unwashed samples. The selected OTUs exhibit mixed responses in Chloroflexi, Firmicutes and Proteobacteria. ID labels 1 through 31 are listed and identified in Table 6.

| Label | OTU | Phylum | Legend | OTU | Phylum |
|-------|---------|----------------|--------|---------|----------------|
| 01 | 4373459 | Acidobacteria | 15 | 573135 | Proteobacteria |
| 02 | 1145388 | Chloroflexi | 16 | 622571 | Proteobacteria |
| 03 | 512148 | Actinobacteria | 17 | 4301099 | Proteobacteria |
| 04 | 125045 | Actinobacteria | 18 | 4308371 | Proteobacteria |
| 05 | 4350881 | Actinobacteria | 19 | 108916 | Proteobacteria |
| 06 | 214718 | Actinobacteria | 20 | 924547 | Proteobacteria |
| 07 | 764292 | Firmicutes | 21 | 950606 | Proteobacteria |
| 08 | 42642 | Firmicutes | 22 | 1118561 | Proteobacteria |
| 09 | 336164 | Firmicutes | 23 | 926370 | Proteobacteria |
| 10 | 4401692 | Firmicutes | 24 | 760529 | Proteobacteria |
| 11 | 1100972 | Firmicutes | 25 | 635524 | Proteobacteria |
| 12 | 4316928 | Firmicutes | 26 | 219439 | Proteobacteria |
| 13 | 839282 | Firmicutes | 27 | 1120966 | Proteobacteria |
| 14 | 781576 | Firmicutes | 28 | 7061 | Proteobacteria |
| | | | 29 | 673343 | Proteobacteria |
| | | | 30 | 4439509 | Proteobacteria |
| | | | 31 | 511916 | Proteobacteria |

Table 6. Corresponding identifiers for heatmap tree comparing phylum of washed and unwashed samples (Associated with Figure 20.)

Label numbers correspond with inside colored phylum ring of the heatmap in Figure 20 and are identified by their OTU and phylum name.

3.4.4. Analysis of gut-associated bacteria with washed and unwashed *Papa kea* samples.

Figure 21. shows the relative abundance of possible human gut bacteria at the family level for RC and RD samples (Filter 5) for the top 150 OTUs. The majority of the bacteria belong to the Family Enterobacteriaceae (54%) and Family Pseudomonadaceae (30%) with the remaining bacteria belonging to families Xanthomonadaceae (6%), Burkholderiaceae (2.7%), Alcaligenaceae (2.0%), Paenibacillaceae (2.0%), Enterococcaceae (1.3%), Comamonadaceae (0.7%), Lachnospiraceae (0.7%) and Streptococcaceae (0.7%).

OTUs (in order of highest to lowest average abundance) 4419276, 133961 (Family: Pseudomonadaceae), 91962, 9994, 676211, 668514, 122049, 233220 and 192342 (Family: Enterobacteriaceae) generally maintained similar abundance between washed (RC) and unwashed samples (RD). A few OTUs demonstrated a dramatic decrease of gut-associated bacteria in washed samples while present in unwashed

samples OTUs (in order of highest to lowest difference in the mean) 394796 (*Pseudomonas viridiflava* Burkholder), 410048 (Genus: *Pseudomonas*), 924547 (Family: Enterobacteriaceae), 4416113 (Family: Enterobacteriaceae, Genus: *Serratia*), 802064 and 1981302 (Family: Burkholderiaceae, Genus: *Burkholderia*), 691423 and 688934 (Family Enterobacteriaceae). On the other hand, one OTU in the Family Enterobacteriaceae (539107) was dramatically higher in abundance in washed samples than unwashed samples suggesting selectivity by the roots for this particular bacterium. Other OTUs were considerably higher in abundance for washed samples (RC) than unwashed samples (RD), specifically OTUs 219439 (Family: Xanthomonadaceae, *Stenotrophomonas geniculata* (Wright)), 4451011 and 544313 (Family: Pseudomonadaceae) while the Family Pseudomonadaceae, OTUs 4364813, 2534143 (Genus: *Pseudomonas*) showed a moderate increase in abundance.

In summary, there is some variability in the abundance of family selectivity among washed and unwashed samples, but more importantly, an overall look at the heatmap shows traditional washing does not remove most of the bacteria keeping the root microbiome intact and suggesting that some of these organisms exist within the root interior.

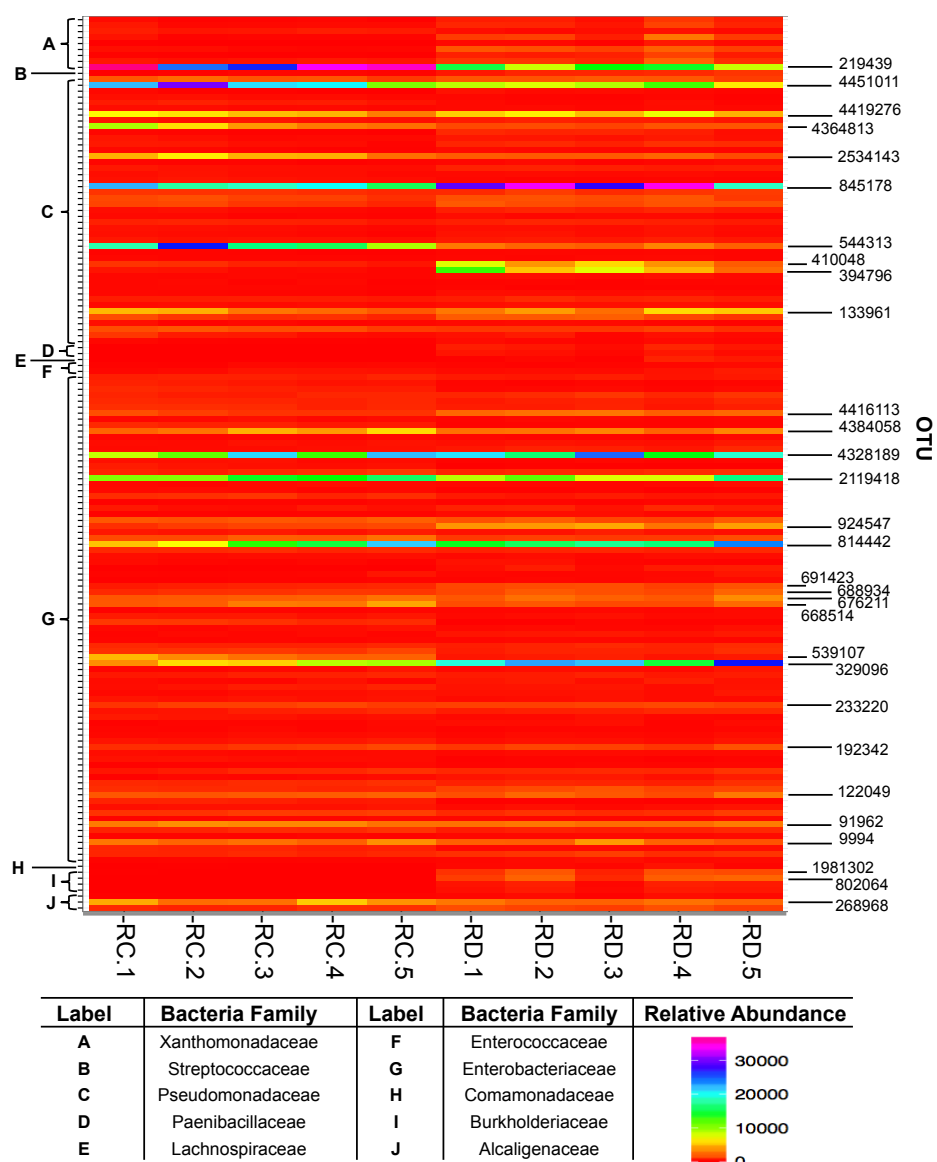


Figure 21. Heatmap to family level comparing relative abundance of gut bacteria in washed and unwashed samples.

From the 13,333 OTUs present in the study, the top 150 OTUs in families associated as possible gut bacteria were compared for relative abundance and selectivity between washed and unwashed samples. Rainbow scale starting with magenta represents abundance above 30000 with red indicating absence of OTU and color hues from blue to orange decrease in abundance. OTUs are labeled on the left vertically by bacteria family with each line representing a separate OTU: Xanthomonadaceae (A), Streptococcaceae (B), Pseudomonadaceae (C), Paenibacillaceae (D), Lachnospiraceae (E), Enterococcaceae (F), Enterobacteriaceae (G), Comamonadaceae (H), Burkholderiaceae (I) and Alcaligenaceae (J). Specific OTU numbers are labeled on the right indicating OTUs of interest. Samples are listed horizontally with in the following order: RC.1, RC.2, RC.3, RC.4, RC.5, RD.1, RD.2, RD.3, RD.4 and RD.5.

3.4.5. Analysis of probiotic and pathogen content of washed and unwashed *Papa kea* samples.

Kava TPK suggests some indications that are ascribed in the modern nutraceutical industry to probiotic agents. We therefore asked if there was any evidence for probiotic microbial communities associated with *Papa kea*, namely families containing the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus* [106]. Out of the 150 top OTUs in Figure 22., probiotic families Streptococcaceae, represent by six OTUs (4468805, 1100972, 544419, 335256, 316321, 294254, respectively), and Lactobacillaceae with two OTUs [781576 (*Lactobacillus manihotivorans* Morlon-Guyot *et al.*) and 291816 (Genus: *Lactobacillus*)] with such low relative abundances that they lack any distinction on the heatmap. More importantly, these bacteria were present mostly in unwashed samples and completely eliminated on washed roots indicate that probiotic bacteria do associate with kava but are largely removed by the particular washing process employed by the practitioners involved in this study. Figure 22. also revealed seven additional bacterial OTUs (labeled in red), four of which, OTUs 68617 (Family: Alcaligenaceae, Genus: *Achromobacter*), 818602, 821562, 4456889 (Family: Pseudomonadaceae, Genus: *Pseudomonas*), all of which showed a decrease in abundance in washed samples compared to unwashed samples and are known plant or human/animal pathogens, so minimally traditional washing does remove pathogenic species.

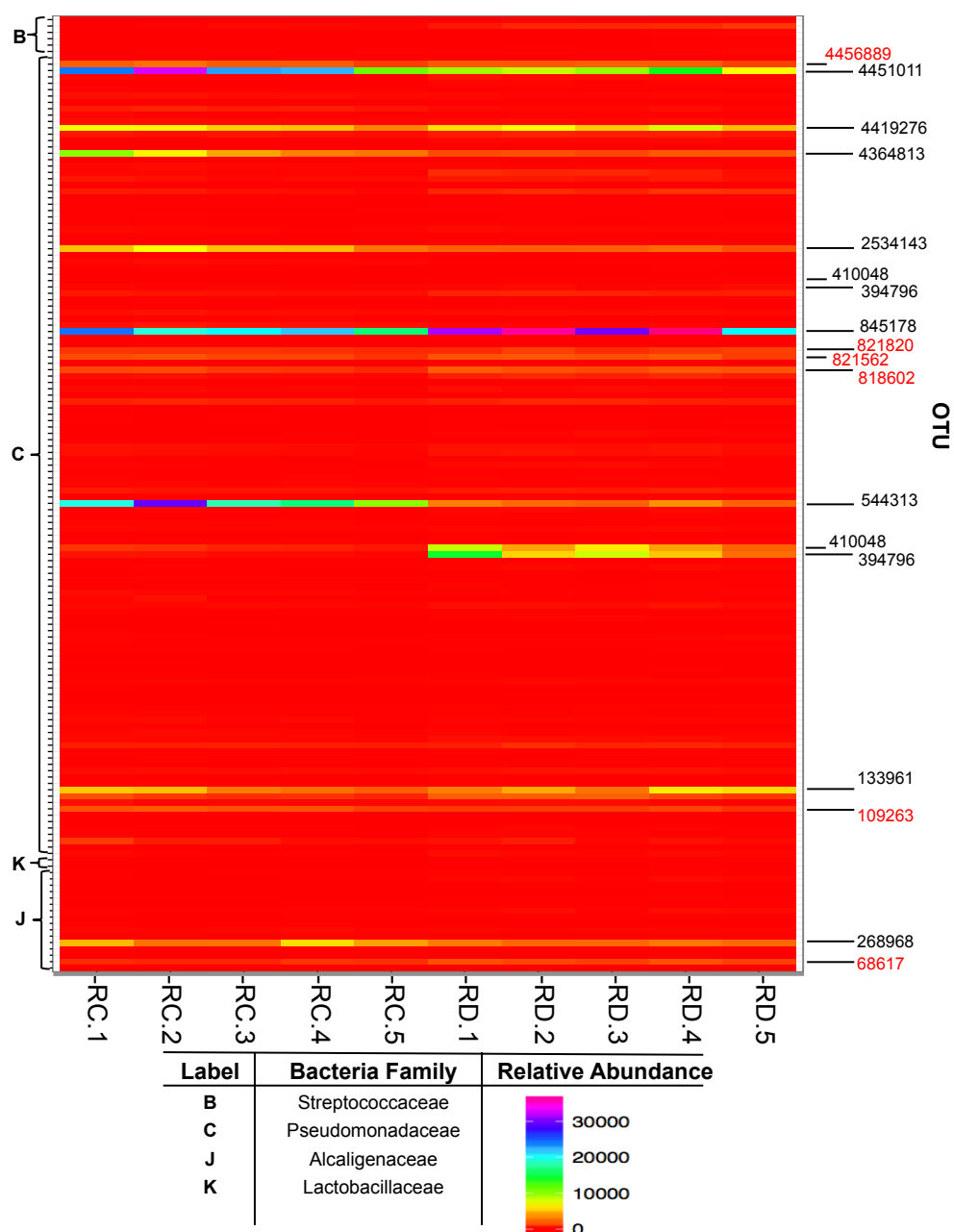


Figure 22. Heatmap to family level comparing abundance of possible probiotic bacteria in washed and unwashed samples.

From the 13,333 OTUs present in the study, the top 150 OTUs in families associated with probiotic bacteria were compared for relative abundance and selectivity between washed and unwashed samples. Rainbow scale starting with magenta represents abundance above 30000 with red indicating absence of OTU and color hues from blue to orange decreasing in abundance. OTUs are labeled vertically by bacteria family with each line representing a separate OTU: Streptococcaceae (**B**), Pseudomonadaceae (**C**), Alcaligenaceae (**J**) and Lactobacillaceae (**K**). Samples are listed horizontally with in the following order: RC.1, RC.2, RC.3, RC.4, RC.5, RD.1, RD.2, RD.3, RD.4 and RD.5.

3.4.6. Comparison between *Hanakapi'ai* root samples and bulk soil

Of 15,617 OTUs, 1,895 displayed abundance patterns with significant differences between HC and HS (Filter 5: taxa with significant abundance differences in one category compared to alternate categories using ANOVA). Figure 23. shows that both qualitative and quantitative indices of alpha diversity generally indicate significant separation between HC and HS with the exception of HS.2, which shows more similarity with HC samples along all indices and as a result, Fisher alpha diversity makes HC.3 appear closer in diversity to soil with root samples with the remaining indices showing at least a moderate separation. Samples HC.2 appears on the outside of the ordination clusters compared to other HC samples but remains significantly separated from all HS samples for all indices except Inverse Simpson, which indicates a closer correlation between HC.2 and HS.2.

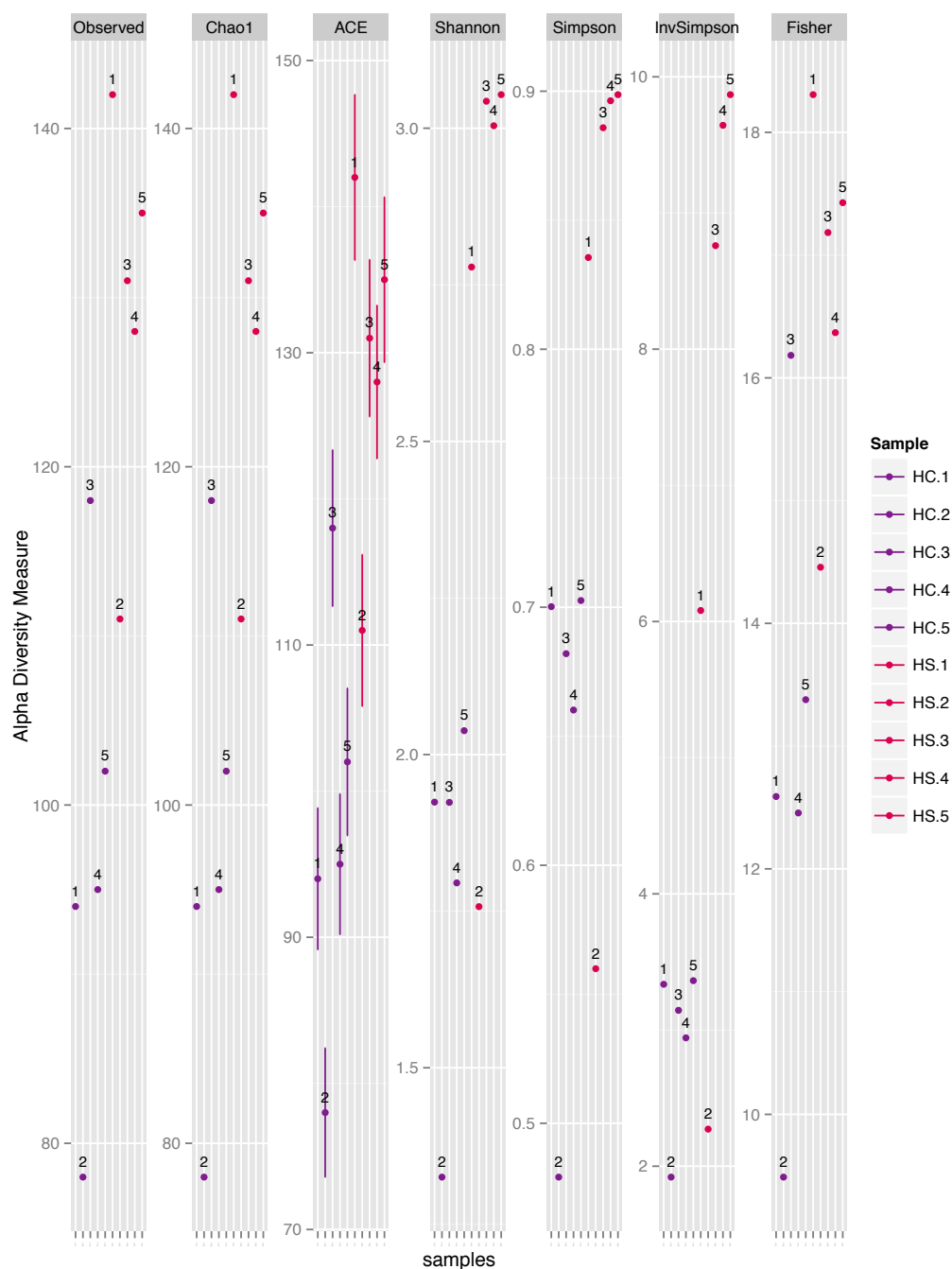


Figure 23. Ordination of HC and HS comparison.

We considered alpha diversity of samples given abundance of 1,895 taxa with significant abundance across at least on of the categories (Filter 5). Non-parametric, qualitative indices used: Chao1 and ACE; Non-parametric, quantitative indices used: Shannon, Simpson, Inverse Simpson and Fisher. Horizontal axis from left to right HC.1, HC.2, HC.3, HC.4, HC.5, HS.1, HS.2, HS.3, HS.4, HS.5 for each indices. Abbreviations are HC.1-5: *Hanakapi'ai* bulk control soil ~ 1 meter from roots and HS.1-5: *Hanakapi'ai* roots with soil.

Detrended Correspondence Analysis (DCA) analysis (Figure 24.) shows a total variance of 89.6% (Axis 1: 72.3% and 17.3%). It was also noted that HS.2 is inconsistent with other samples appearing more like *Hanakapi'ai* bulk control soil samples on axis 2, and more similar to HC.2 from the y-axis perspective. HC.5 also shows some dissimilarity on the y-axis compared to the main cluster; however, ordination plots across all indices indicated similarity of this sample to the remaining cluster. This result is consistent with the results seen in Figure 23.; however, it does show notable difference in variation to bulk soil samples not easily depicted in the alpha diversity plots. Hierarchical Clustering (Figure 25.) revealed distinct clustering of HC and HS samples.

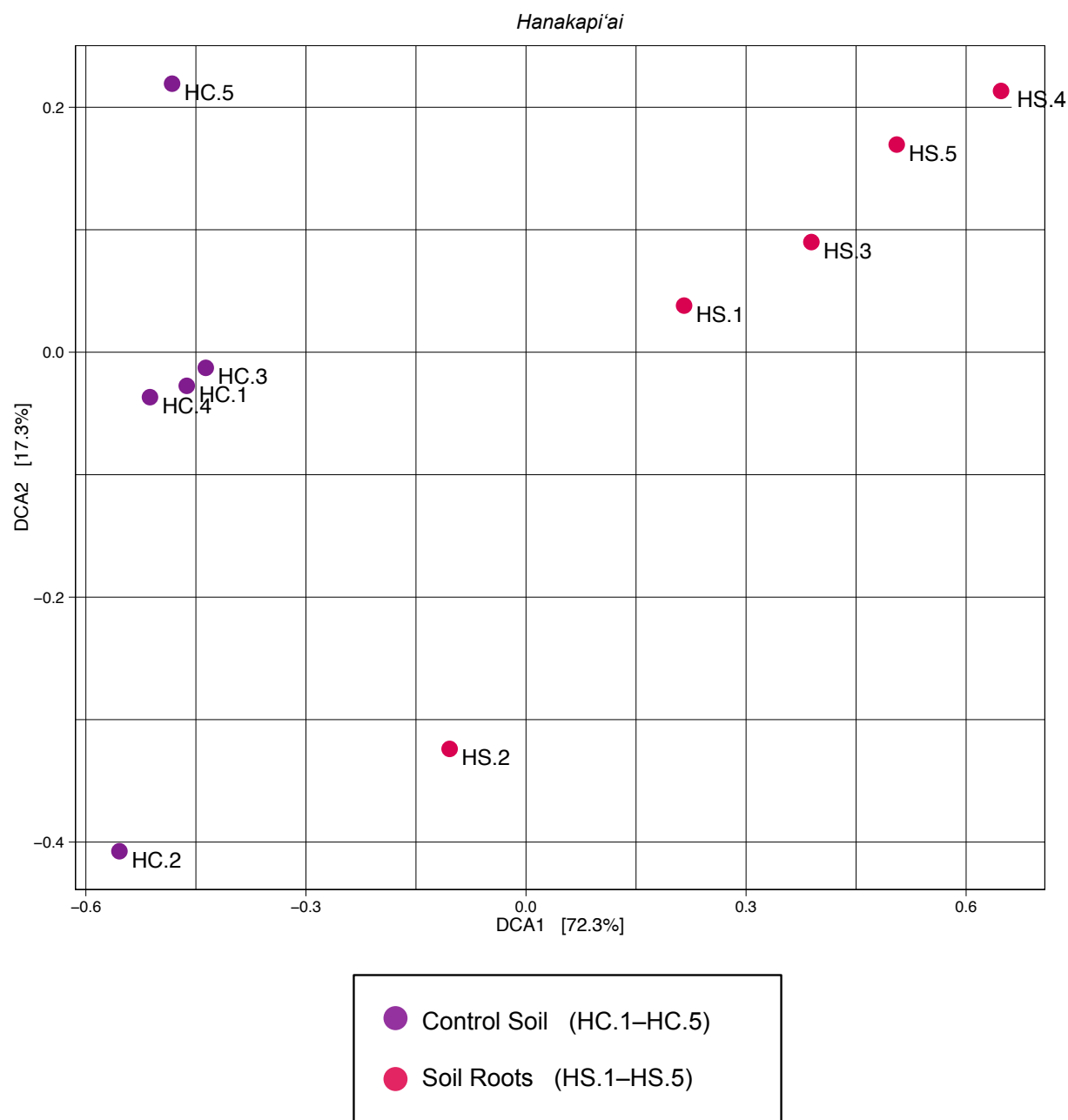


Figure 24. HS vs HC DCA based on weighted UniFrac.

Detrended Correspondence Analysis (DCA) based on weighted UniFrac distance based on Weighted UniFrac distance between samples given abundance of 1,895 taxa with significant abundance differences across at least one of the categories. Abbreviations are HC.1-5: *Hanakapi'ai* bulk control soil ~ 1 meter from roots and HS.1-5: *Hanakapi'ai* roots with soil.

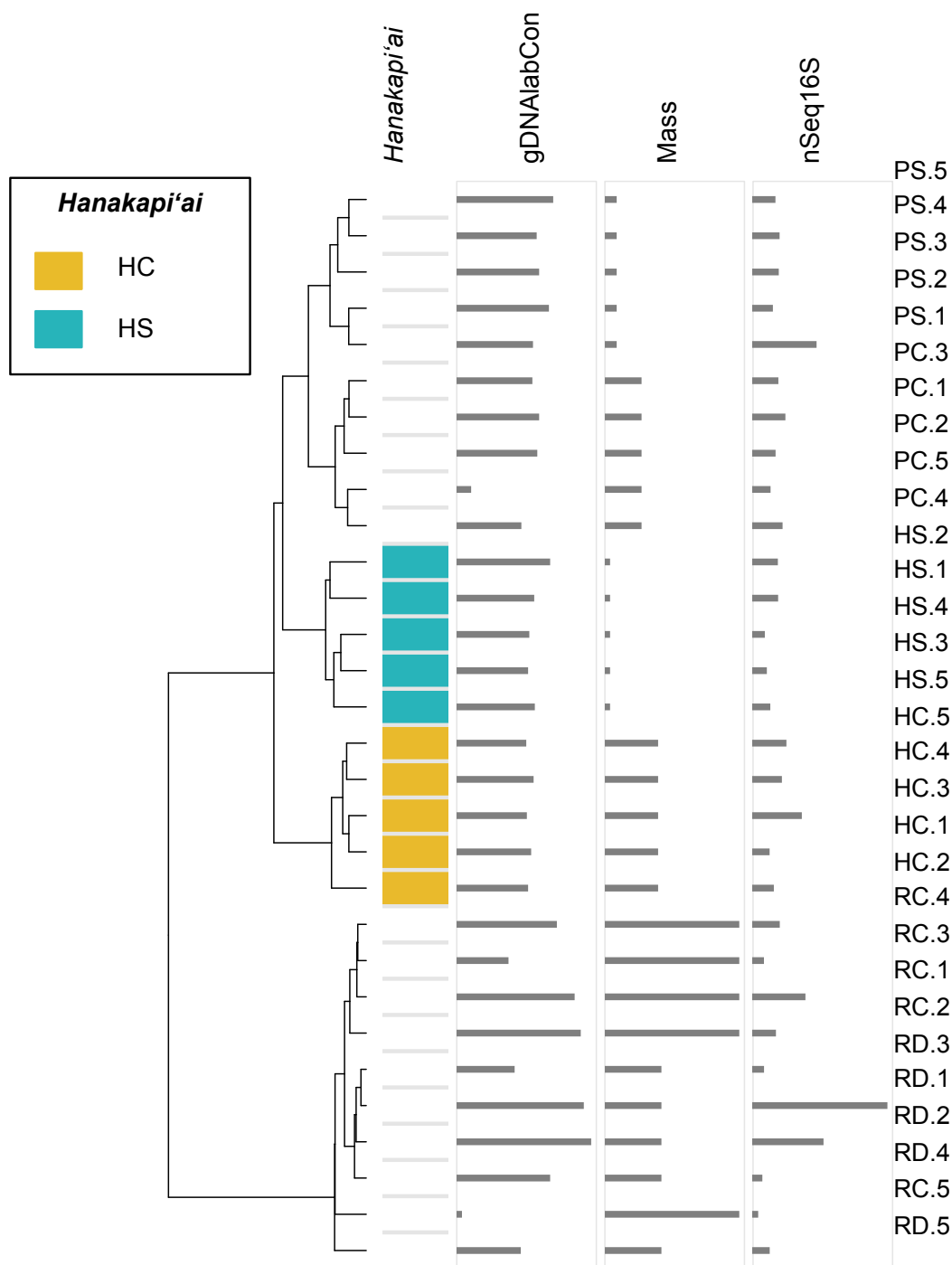


Figure 25. HS vs HC hierarchical clustering weighted.

Based on Weighted UniFrac distance between samples given abundance of 1,895 taxa with significant abundance differences across at least one of the categories. Using 1,895 at5 filtered taxa, the microbiome community characterization of samples from HC and HS revealed distinct cluster. Abbreviations are HC.1-5: *Hanakapi'ai* bulk control soil ~ 1 meter from roots and HS.1-5: *Hanakapi'ai* roots with soil.

Figure 26. (with identifications of OTUs on Table 7) outlines the profiles of OTUs that generate the lowest p-values; OTUs in the top 12 belong to three phyla Proteobacteria (9), Bacteroidetes (2) and Actinobacteria (1). There was significant difference between OTUs selected for by HS samples compared to bulk soil samples, but more importantly, abundance was significantly reduced for 5 OTUs: 548754 and 4377315 (Proteobacteria: Moraxellaceae), 143093 (Proteobacteria: Gammaproteobacteria), 590507 (Proteobacteria: Alphaproteobacteria and 1095594 (Actinobacteria: Micromonospraceae)

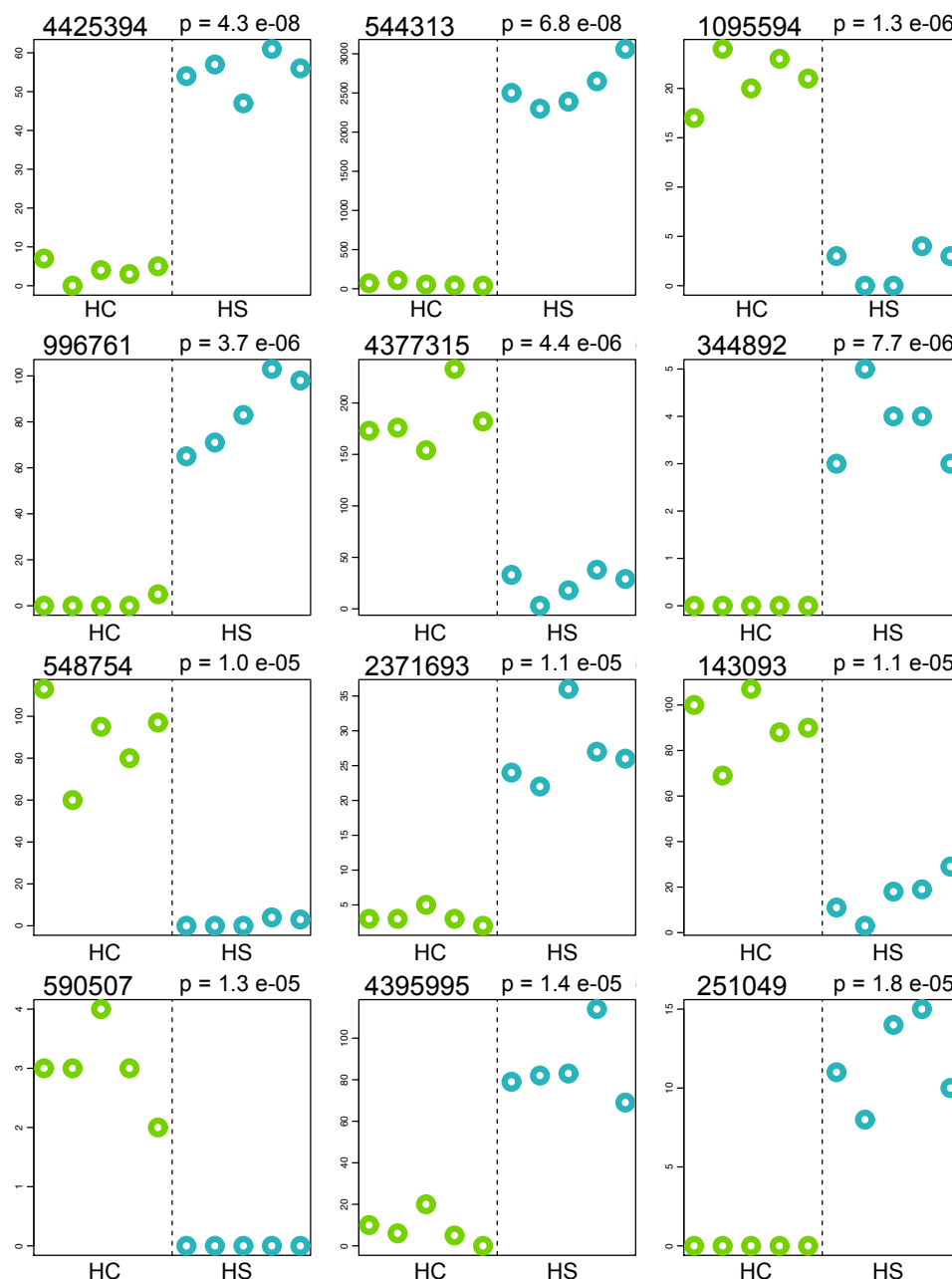


Figure 26. HS vs HC profiles of at5 OTUs generating the lowest p-values.

P-values shown at top of each OTU plot are unadjusted for multiple testing. The y-axis represents the OTU abundance. *Hanakapi* 'ai samples are grouped and colored by category along the x-axis in the following order: HC.1, HC.2, HC.3, HC.4, HC.5, HS.1, HS.2, HS.3, HS.4 and HS.5. All selected OTUs of the top 12 belonged to one of 3 phyla: Proteobacteria (9), Bacteroidetes (2) and Actinobacteria (1). Seven of 12 selected OTUs displayed a significant increase in HS samples, and OTU 1095594 (Micromonosporaceae) was significantly greater in all HC samples.

| | OTU | Phylum | Class | Order | Family | Genus | Species |
|--------|---------|----------------|---------------------|--------------------|---------------------|--------------------|--------------|
| Left | 4425394 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Pseudomonadaceae | <i>Pseudomonas</i> | unclassified |
| | 996761 | Bacteroidetes | Sphingobacteria | Sphingobacteriales | Sphingobacteriaceae | unclassified | unclassified |
| | 548754 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | 94otu37979 | 97otu50624 |
| | 590507 | Proteobacteria | Alphaproteobacteria | 85otu312 | 91otu812 | 94otu1588 | 97otu11171 |
| Middle | 544313 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Pseudomonadaceae | <i>Pseudomonas</i> | unclassified |
| | 4377315 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | unclassified | unclassified |
| | 2371693 | Proteobacteria | Betaproteobacteria | unclassified | unclassified | unclassified | unclassified |
| | 4395995 | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | unclassified | unclassified |
| Right | 1095594 | Actinobacteria | Actinobacteria | Actinomycetales | Micromonosporaceae | 94otu8918 | 97otu66145 |
| | 344892 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | unclassified | unclassified |
| | 143093 | Proteobacteria | Gammaproteobacteria | 34P16 | 91otu11085 | 94otu21318 | unclassified |
| | 251049 | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | 91otu4379 | unclassified | unclassified |

Table 7. HS vs HC annotations of the OTUs with the lowest p-values.

Corresponding taxa ID number of the top 12 phyla (associated with Figure 26) identified to Class, Order, Family, Genus and Species where possible. OTUs are listed from the left, middle and right, then top to bottom as depicted in Figure

Figure 27. (a. and b.) bacteria heatmap with identification listed in Table 8 shows 33 families in the top 150 OTUs of the 15,617 OTUs present compared for relative abundance and selectivity between *Hanakapi'ai* bulk soil (HC) and *Hanakapi'ai* roots (HS) with rainbow scale starts with magenta representing higher relative abundance and color hues from blue to red-orange decreasing in abundance. We \log_2 transformed the data set to more closely align scaling between *Hanakapi'ai* and *Papa 'ele 'ele* for comparison of Family groups and OTUs between these two cultivars; however, the below analysis was based on relative abundance values. Of the top 150 OTUs, the Family Hyphomicrobiaceae had the largest percentage at 24% with the second largest Family Gaiellaceae (8.7%) followed by Sinobacteraceae and Xanthomondaceae (6.7% each) and Koribacteraceae and Solibacteriaceae (5.3% each) with the remaining bacterial families each making up less than 5% and some families only represented by only one or two OTUs. Overall, these results indicate that there is a large diversity among both

Hanakapi'ai bulk soil and *Hanakapi'ai* roots, though abundance for many OTUs was variable across most HC and HS samples. An overall look at the heatmap indicates that samples HC.2 and HS.2 are generally inconsistent with other bulk soil and root with soil samples, respectively with HS.2 being more significantly different than the other samples in that category (see discussion). As a result, some OTUs were difficult to determine the trend specifically: 1129425 (Solirubrobacteraceae: *Solirubrobacter*), 4298129 (Rhodospirillaceae), 994823 (Patulibacteraceae), 1110792 (Koribacteraceae: *Candidatus Koribacter*), 253167 and 52053 (Hyphomicrobiaceae: *Rhodoplanes*), and 1624 (Chthoniobacteraceae: DA101).

Outside of these issues, strong relative abundances were seen in all samples (HC and HS) for several OTUs with some exceptions in the Families Xanthomonadaceae (1118758); Sinobacteraceae (252012 except HS.5 was slightly lower); Hyphomicrobiaceae (4431597, 4342107 and 1657331); Hyphomicrobiaceae identified to Genus *Rhodoplanes* (248032, 156044, 136162 and 101542) and Bradyrhizobiaceae: Genus *Bradyrhizobium* (4475561 and 740317).

Of more importance is the potential ability of the *Hanakapi'ai* cultivar to select for specific bacteria, despite lower abundances in bulk soil. The family heatmap revealed that at least 17 OTUs demonstrated some level of selectivity indicated by higher abundances around root with soil samples. Strong selectivity was noted in OTUs in the Families Xanthomonadaceae (434357, 707290, 184325 and 34580), Sphingomonadaceae (243129), Sphingobacteriaceae (357684), Pseudomonadaceae (845178 and 133961), and Comamonadaceae (4453710). Relatively strong selectivity for one identified as Nitrospiraceae: *Nitrospira* (89259).

Another expectation was that relatively high abundance in bulk soil populations would be similar in root with soil samples due to their higher presence in the surrounding soil. In contrast, our results suggested that some OTUs actually declined in abundance around (HS samples), which we believe may be an example of de-selectivity by roots. In fact, as many as 31 OTUs demonstrated this de-selectivity by higher abundances found in bulk soil samples (HC) compared to roots (HS). *Hanakapi'ai* bulk soil (HC) samples showed higher relative abundance for some OTUs compared to roots samples (HS): OTU

81089 (Xanthomonadaceae), 13034 (Mycobacteriaceae: *Mycobacteriaceae*), 235789 and 53533 (*Rhodoplanes*) and higher abundance in most bulk soil samples in two OTUs in the family Sinobacteraceae: 4373617 and 4353076 (*Steroidobacter*) with some variability in the relative abundance. OTU 53533 trend for de-selectivity remained strong with and without HS.2 being higher in abundance than other HS samples.

| Label | Bacteria | Label | Bacteria |
|-------|----------------------|-------|---------------------|
| 1 | Xanthomonadaceae | 18 | Micromonosporaceae |
| 2 | Syntrophobacteraceae | 19 | Koribacteraceae |
| 3 | Sphingomonadaceae | 20 | Hyphomonadaceae |
| 4 | Sphingobacteriaceae | 21 | Hyphomicrobiaceae |
| 5 | Solirubrobacetaceae | 22 | Haliangiaceae |
| 6 | Solibacteraceae | 23 | Gaiellaceae |
| 7 | Sinobacteraceae | 24 | Enttheonellaceae |
| 8 | Rhodospirillaceae | 25 | Cytophagaceae |
| 9 | Pseudonocardiaceae | 26 | Conexibacteraceae |
| 10 | Pseudomonadaceae | 27 | Comamonadaceae |
| 11 | Piscirickettsiaceae | 28 | Chthoniobacteraceae |
| 12 | Phyllobacteriaceae | 29 | Chitinophagaceae |
| 13 | Patulibacteraceae | 30 | Burkholderiaceae |
| 14 | Nocardioideae | 31 | Bradyrhizobiaceae |
| 15 | Nitrospiraceae | 32 | Acidobacteriaceae |
| 16 | Nitrosomonadaceae | 33 | Acetobacteraceae |
| 17 | Mycobacteriaceae | | |

Table 8. HS vs. HC annotations bacteria family heatmap.

Numerical label with corresponding Bacterial family identification (associated with Figure 27.).

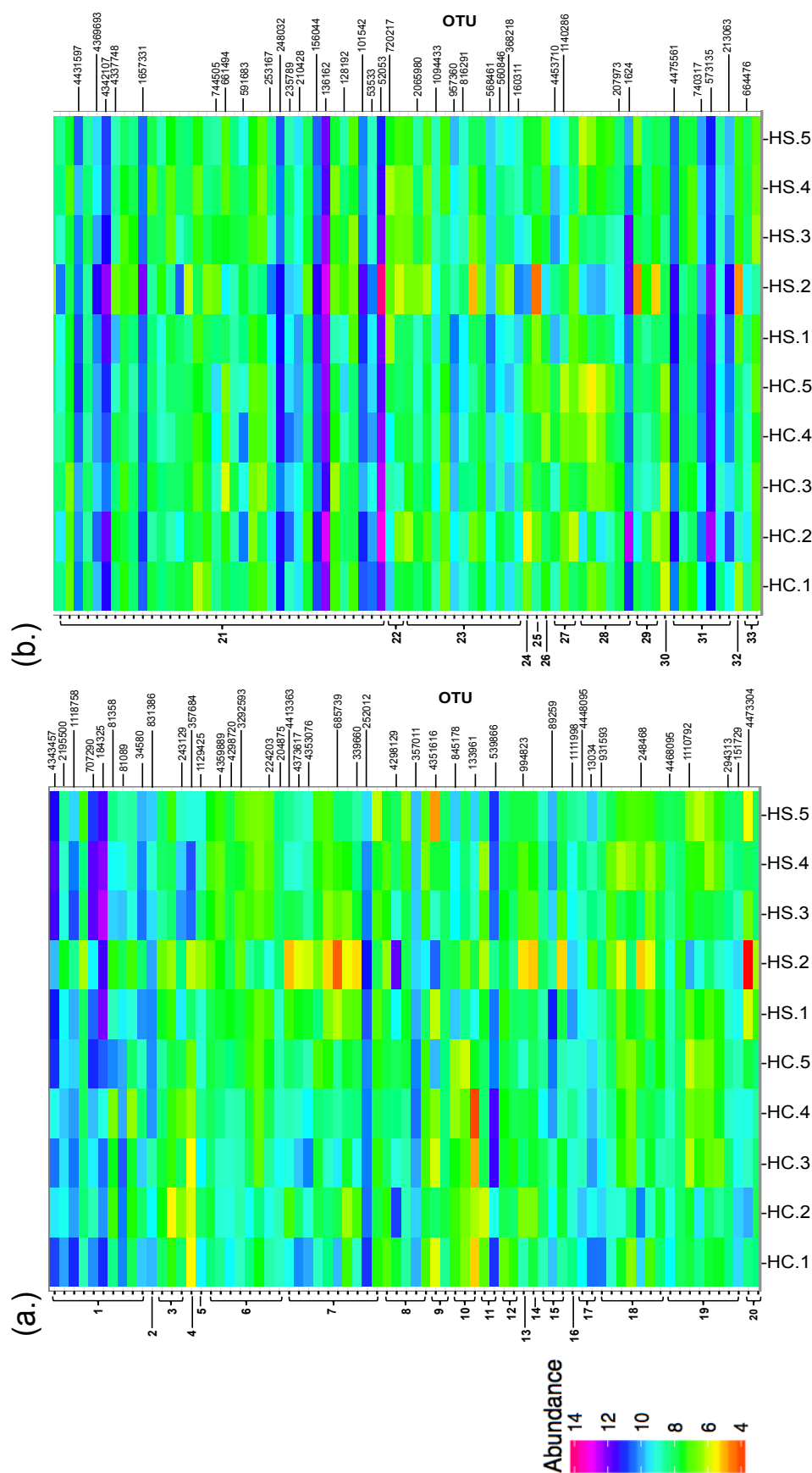


Figure 27. (a.) and (b.) HS vs. HC bacteria family heatmap. From the 15,617 OTUs present in the study, the top 150 OTUs covering 33 families were compared for abundance and selectivity between *Hanakapi'ai* bulk soil and *Hanakapi'ai* roots. Rainbow scale starting with magenta represents higher relative abundance with color hues from blue to red-orange decreasing in abundance; data set was \log_2 transformed to more closely align scaling between *Hanakapi'ai* and *Papa 'ele 'ele* for comparison. OTUs are labeled vertically on the left axis by bacteria family with each line representing a separate OTU; annotations of Label number and Family identification are listed on Table 8. Specific OTU numbers are labeled on the right indicating OTUs of interest. Samples are listed horizontally with in the following order: HC.1, HC.2, HC.3, HC.4, HC.5, HS.1, HS.2, HS.3, HS.4 and HS.5.

3.4.7. Comparison between *Papa 'ele 'ele* roots and bulk soil

Similar analysis of *Papa 'ele 'ele* was conducted based on 15,617 OTUs with 2,796 displayed abundance patterns with significant differences between PC and PS (Filter 5: taxa with significant abundance differences in one category compared to alternate categories using ANOVA), which also demonstrated separation and distinct clustering between bulk soil and root samples. Figure 28. shows that both qualitative and quantitative indices of alpha diversity with PC samples generally well grouped while PS samples are more spread out; however, moderate separation is noted between PS and PC samples for all indices with the exception of Chao1. The Chao 1 index, which is based upon the number of rare OTUs found in a sample [107], shows PS.2, PS.3, PS.4, and PS.5 more similar to PC samples with PS.1 showing the greatest separation but with large error bars. ACE shows some similarity to the results in Chao1 indices, but appears a bit more separated. Both Chao and ACE are known to underestimate true richness in small sample sizes such as those used in this study [108].

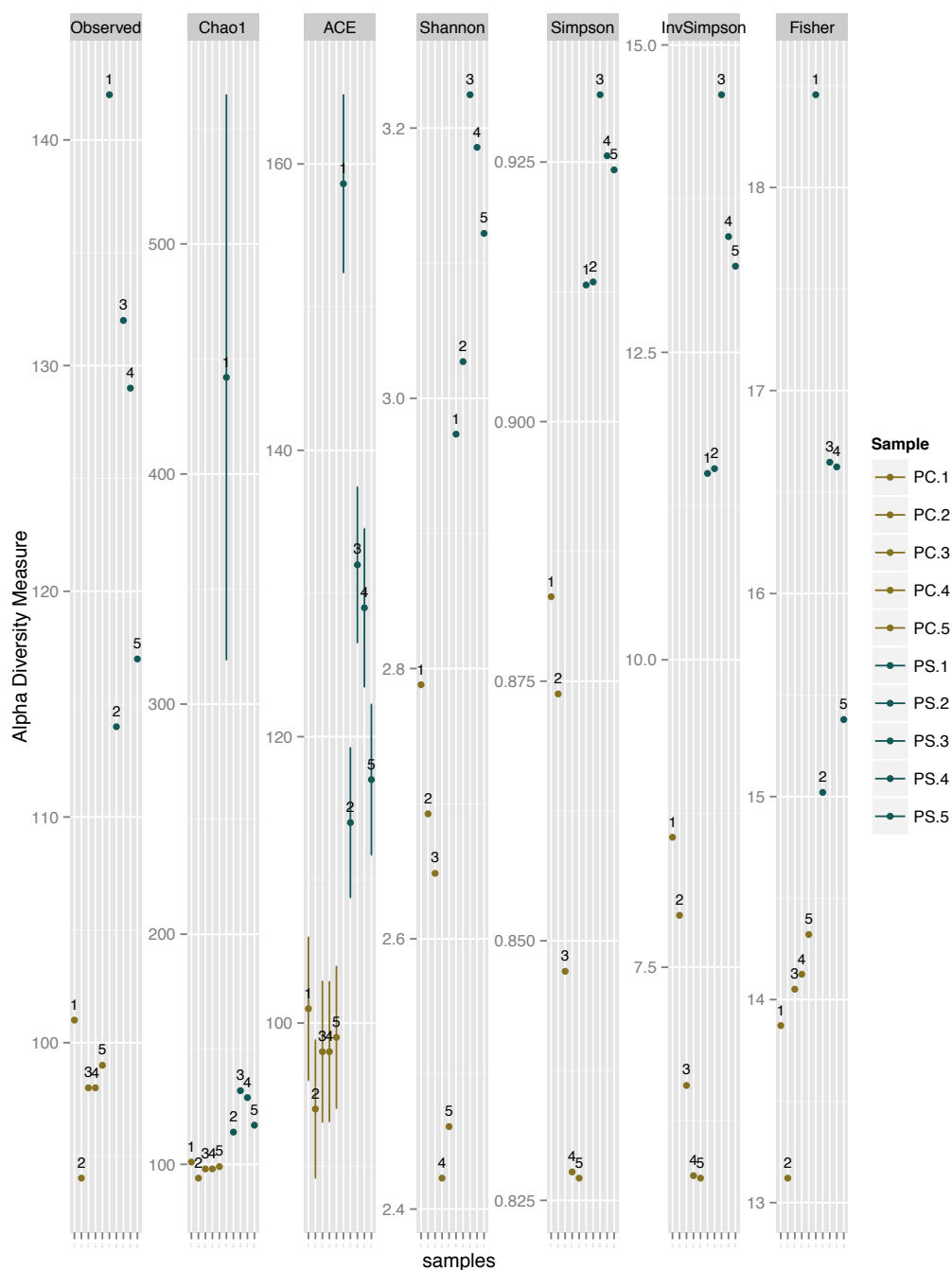


Figure 28. Ordination of PC and PS comparison.

We considered alpha diversity of samples given abundance of 2,796 taxa with significant abundance across at least on of the categories (Filter 5). Non-parametric, qualitative indices used: Chao1 and ACE; Non-parametric, quantitative indices used: Shannon, Simpson, Inverse Simpson and Fisher. Horizontal axis from left to right PC.1, PC.2, PC.3, PC.4, PC.5, PS.1, PS.2, PS.3, PS.4, PS.5 for each indices. Abbreviations are PC.1-5: washed *Papa 'ele 'ele* bulk control soil ~ 1 meter from roots and PS.1-5: *Papa 'ele 'ele* roots.

Detrended Correspondence Analysis (DCA) analysis (Figure 29.) shows a total variance of 86.7% (Axis 1: 70.9% and 15.8%), and more clearly shows the separation between PC and PS samples including PS.1 being more consistent with PS.2 and PS.5 samples. Hierarchical Clustering (Figure 30.) revealed distinct clustering of PC and PS samples. Figure 31. (with identifications of OTUs on Table 9) outlines the profiles of OTUs that generate the lowest p-values; OTUs in the top 12 belong to five phyla Proteobacteria (8), Nitrospirae (1), Planctomycetes (1), Acidobacteria (1) and Bacteroidetes (1). OTU 4335077, genus *Burkholderia*, was significantly greater in all PS samples. OTU 56109, class Betaproteobacteria and OTU 731730, class Acidobacteria-6, were also greater in abundance in PS samples compared to PC samples and OTU 4026017, family Rhodospirillaceae showed some increase in abundance in all PS samples.

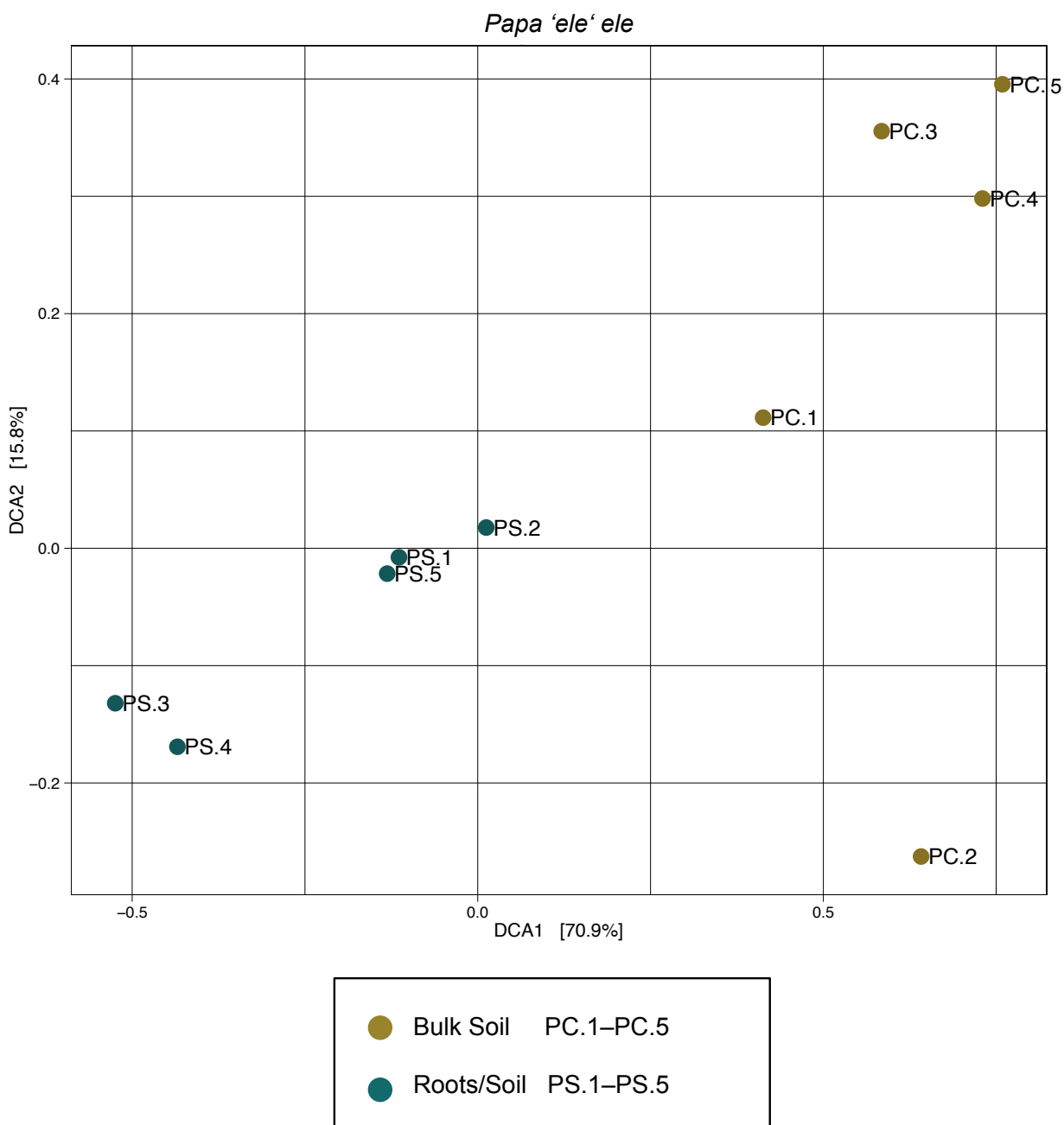


Figure 29. PS vs. PC DCA based on weighted UniFrac.

Detrended Correspondence Analysis (DCA) based on weighted UniFrac distance based on Weighted UniFrac distance between samples given abundance of 2,796 taxa with significant abundance differences across at least one of the categories.

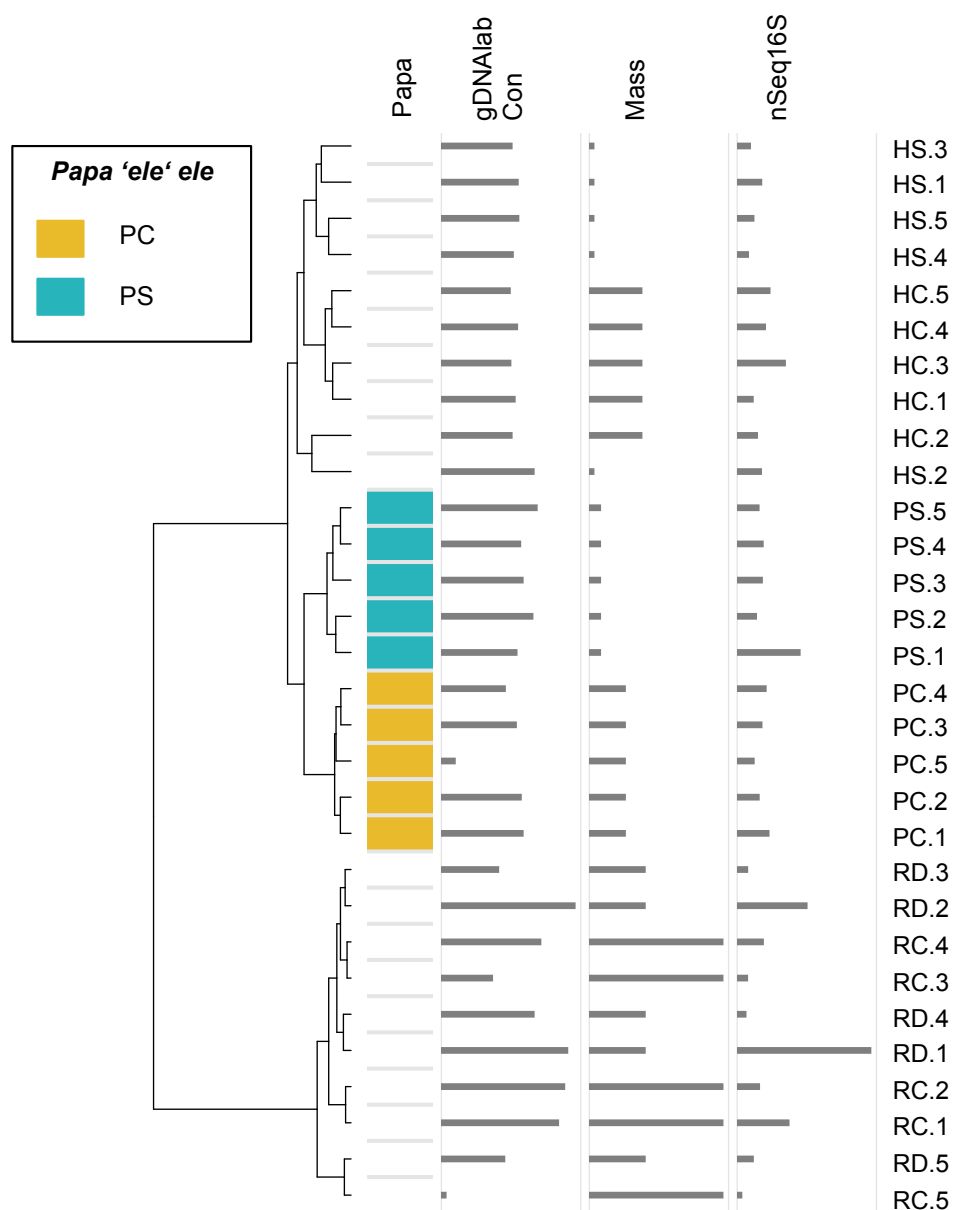


Figure 30. PS vs. PC hierarchical clustering weighted (average linkage).

Based on Weighted UniFrac distance between samples given abundance of 2,796 taxa with significant abundance differences across at least one of the categories. Using 2,796 at5 filtered taxa, the microbiome community characterization of samples from PC and PS revealed distinct cluster. Abbreviations are PC.1-5: washed *Papa 'ele 'ele* bulk control soil ~ 1 meter from roots and PS.1-5: *Papa 'ele 'ele* roots.

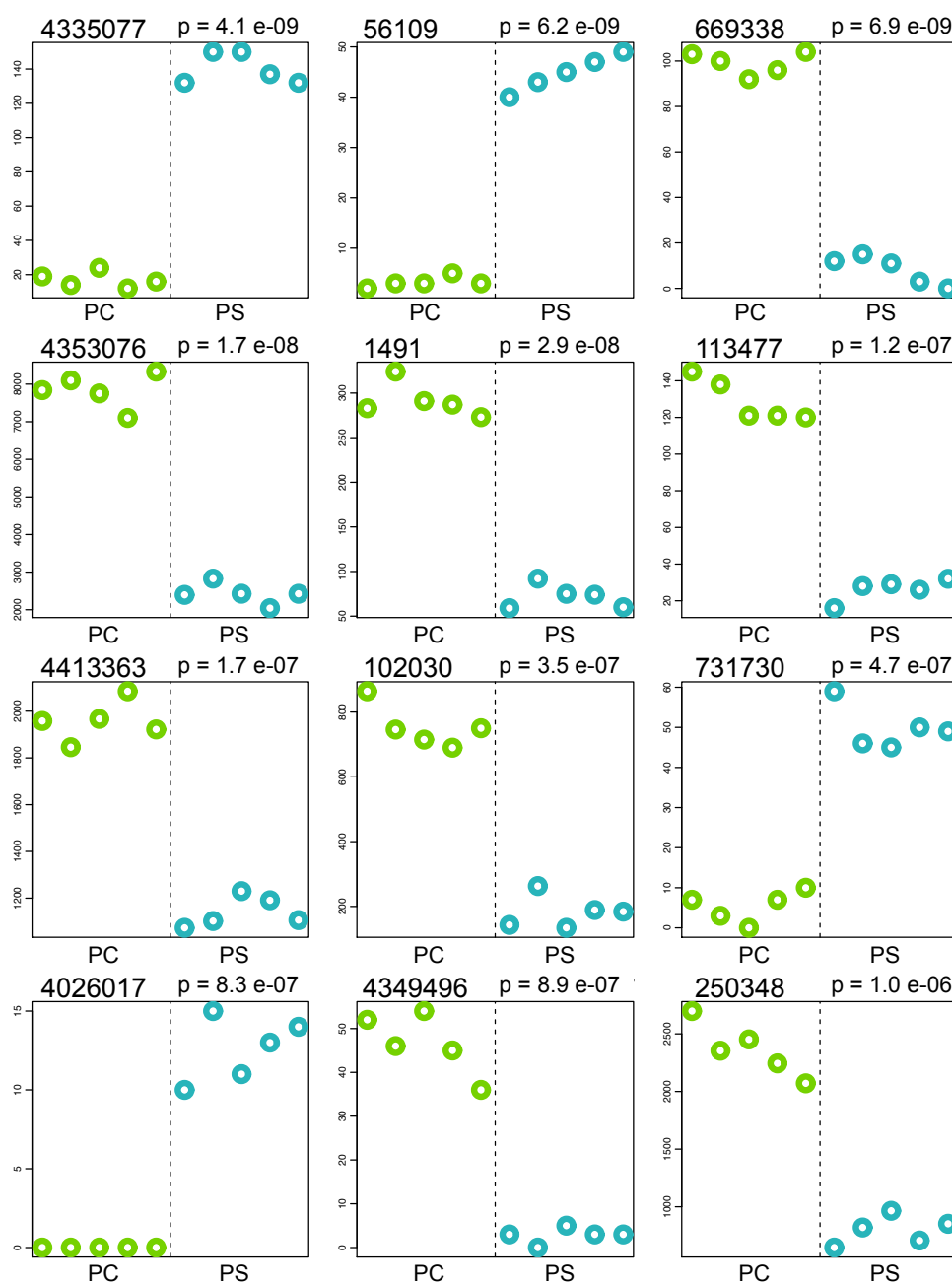


Figure 31. PS vs. PC profiles of at5 OTUs generating the lowest p-values.

P-values shown at top of each OTU plot are unadjusted for multiple testing. The y-axis represents the OTU abundance. Samples are grouped and colored by category along the x-axis in the following order: PC.1, PC.2, PC.3, PC.4 PC.5, PS.1, PS.2, PS.3, PS.4 and PS.5. All selected OTUs of the top 12 belonged to one of 5 phyla: Proteobacteria (8), Nitrospirae (1), Planctomycetes (1), Acidobacteria (1) and Bacteroidetes (1). Eight of 12 selected OTUs displayed a significant increase in PC samples, and OTU 4335077 (Genus: Burkholderia) was significantly greater in all PS samples.

| | OTU | Phylum | Class | Order | Family | Genus | Species |
|--------|---------|----------------|---------------------|------------------|-------------------|-----------------------|--------------|
| Left | 4335077 | Proteobacteria | Betaproteobacteria | Burkholderiales | Burkholderiaceae | <i>Burkholderia</i> | unclassified |
| | 4353076 | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Sinobacteraceae | <i>Steroidobacter</i> | 97otu98065 |
| | 4413363 | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Sinobacteraceae | 94otu40427 | 97otu98093 |
| | 4026017 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | unclassified | unclassified |
| Middle | 56109 | Proteobacteria | Betaproteobacteria | 85otu2071 | 91otu4711 | 94otu13351 | unclassified |
| | 1491 | Nitrospirae | Nitrospira | Nitrospirales | Nitrospiraceae | <i>Nitrospira</i> | 97otu99102 |
| | 102030 | Proteobacteria | Alphaproteobacteria | Ellin329 | 91otu10045 | 94otu19605 | unclassified |
| | 4349496 | Bacteroidetes | [Saprospirae] | [Saprospirales] | Saprospiraceae | unclassified | unclassified |
| Right | 669338 | Planctomycetes | Planctomycetia | Pirellulales | Pirellulaceae | 94otu41539 | 97otu46585 |
| | 113477 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhizobiaceae | unclassified | unclassified |
| | 731730 | Acidobacteria | Acidobacteria-6 | iii1-15 | 91otu8728 | 94otu9359 | unclassified |
| | 250348 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | 94otu18807 | unclassified |

Table 9. PS vs. PC annotations of the OTUs with the lowest p-values.

Corresponding taxa ID number of the top phyla (associated with Figure 31) identified to Class, Order, Family, Genus and Species determinations where possible. OTUs are listed from the left, middle and right, then top to bottom as depicted in Figure

We also investigated abundance and selectivity between *Papa 'ele 'ele* bulk soil (PC) and *Papa 'ele 'ele* roots (PS) [Figure 32. (a.) and (b.) bacteria heatmap with identification listed in Table 10.], representing 34 families in the top 150 OTUs of the 15,617 OTUs present. Of the top 150 OTUs, the Family Hyphomicrobiaceae had the largest percentage at 29.3% with the second largest Family Gaiellaceae (13.3%) followed by Comamonadaceae (6%). The remaining bacterial families each made up less than 5% with some families represented by only one or two OTUs also indicating that a large diversity among both *Papa 'ele 'ele* bulk soil and roots exists though abundance for most OTUs was relatively consistent with some variability across many PC and PS samples.

As with *Hanakapi'ai* samples, *Papa 'ele 'ele* contained OTUs with strong relative abundances across all samples; for example, one OTU in the Family Gaiellaceae (368218) and three OTUs identified as Bradyrhizobiaceae: *Bradyrhizobium* (4475561, 740317 and 573135) as well as in OTUs 1657331 (Hyphomicrobiaceae: *Pedomicrobium*,

137981 (Micrococcaceae). *Papa 'ele 'ele* samples also suggested selectivity by root samples (PS) with at least 29 OTUs demonstrated some level of selectivity with strong selectivity detected in OTUs 150689 (Sphingomonadaceae: *Sphingobium*), 252822 (Rhizobiaceae: *Agrobacterium*), 4314416 (Methylophilaceae) and three OTUs identified as Pseudomonadaceae: *Pseudomonas* (4451011, 544313 and 133961). We also saw de-selectivity in *Papa 'ele 'ele* root samples (PS) with strong de-selectivity in the OTUSs 4353076 (Sinobacteraceae: *Steroidobacter*), 513355 (Saprospiraceae), 4298215 (Rhodospirillaceae) and 305897 (Nocardiodaceae: *Kribbella*).

| Label | Bacteria | Label | Bacteria |
|-------|----------------------|-------|---------------------|
| 1 | Xanthomonadaceae | 14 | Nocardiodaceae |
| 2 | Syntrophobacteraceae | 15 | Nitrospiraceae |
| 34 | Streptomycetaceae | 17 | Mycobacteriaceae |
| 3 | Sphingomonadaceae | 18 | Micromonosporaceae |
| 4 | Sphingobacteriaceae | 38 | Micrococcaceae |
| 5 | Solirubrobacetaceae | 39 | Methylophilaceae |
| 6 | Solibacteraceae | 20 | Hyphomonadaceae |
| 7 | Sinobacteraceae | 21 | Hyphomicrobiaceae |
| 35 | Saprospiraceae | 23 | Gaiellaceae |
| 8 | Rhodospirillaceae | 40 | Erythrobacteraceae |
| 36 | Rhizobiaceae | 24 | Enttheonellaceae |
| 9 | Pseudonocardiaceae | 25 | Cytophagaceae |
| 10 | Pseudomonadaceae | 27 | Comamonadaceae |
| 11 | Piscirickettsiaceae | 28 | Chthoniobacteraceae |
| 37 | Pirellulaceae | 29 | Chitinophagaceae |
| 12 | Phyllobacteriaceae | 31 | Bradyrhizobiaceae |
| 13 | Patulibacteraceae | 41 | Alcaligenaceae |

Table 10. PS vs. PC annotations bacteria family heatmap.

Numerical label with corresponding Bacterial family identification (associated with Figure 32.).

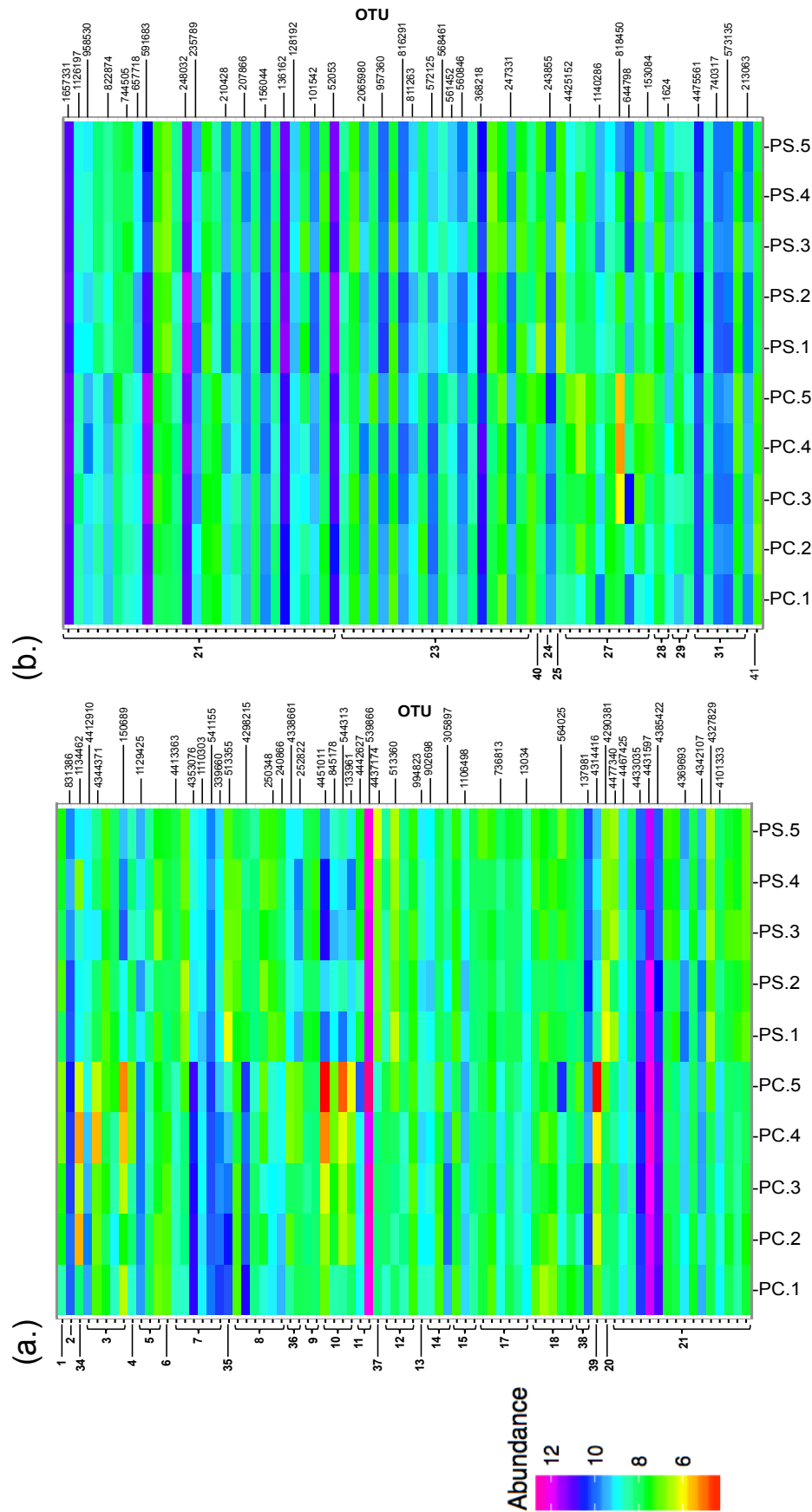


Figure 32. (a.) and (b.) PS vs. PC bacteria family heatmap. From the 15,617 OTUs present in the study, the top 150 OTUs covering 34 families were compared for abundance and selectivity between *Papa 'ele 'ele* bulk soil and *Papa 'ele 'ele* roots. Rainbow scale starting with magenta represents higher relative abundance with color hues from blue to red-orange decreasing in abundance; data set was \log_2 transformed to more closely align scaling between *Papa 'ele 'ele* and *Hanakapi 'ai* for comparison. OTUs are labeled vertically by bacteria family with each line representing a separate OTU; annotations of Label number and Family identification are listed on Table 10. Specific OTU numbers are labeled on the right indicating OTUs of interest. Samples are listed horizontally with in the following order: PC.1, PC.2, PC.3, PC.4, PC.5, PS.1, PS.2, PS.3, PS.4 and PS.5.

3.4.8. Differences between *Hanakapi'ai* and *Papa 'ele 'ele* family relative abundances

Comparison of the family heatmaps revealed distinct differences in bacterial abundance and composition. Overall, *Papa 'ele 'ele* abundance values were generally lower (see log₂ scale), but slightly more diverse than *Hanakapi'ai* samples despite having a higher percentage of Hyphomicrobiaceae. The *Papa 'ele 'ele* cultivar also appears to demonstrate less differences between bulk soil (PC) and root samples (PS) with many OTUs being strongly represented across all samples suggesting that this cultivar is less selective of bacteria species. Compositional difference can be seen in Table 11. and 12. Table 11. list bacteria families that differed in the top 150 OTUs of *Hanakapi'ai* and *Papa 'ele 'ele* cultivars, respectively. *Hanakapi'ai* contained seven different families: Nitrosomonadaceae, Koribacteraceae, Haliangiaceae, Conexibacteraceae, Burkholderiaceae, Acidobacteriaceae and Acetobacteraceae. *Papa 'ele 'ele* contained eight differing families: Streptomycetaceae, Saprospiraceae, Rhizobiaceae, Pirellulaceae, Micrococcaceae, Methyphilaceae, Erythrobacteraceae and Alcaligenaceae.

Even in the case of bacterial families present in both cultivars, there are some differences in total representation by families as well as by specific OTUs representing these families. For starters, by total number of OTUs, Xanthomonadaceae, Solibacteraceae, Chthoniobacteraceae were more heavily represented in *Hanakapi'ai* samples than *Papa 'ele 'ele* samples with Sinobacteraceae and Micromonosporaceae slightly more represented; on the other hand, *Papa* samples were more heavily represent by Hyphomicrobiaceae, Gaiellaceae and Comamonadaceae with Spingomonadaceae, Phyllobacteriaceae and Nocardiodaceae being slightly more represented. Table 12. lists the different OTUs between *Hanakapi'ai* and *Papa 'ele 'ele* samples in shared families; consequently, all of the Xanthomonadaceae OTUs in *Hanakapi'ai* samples differed from the one OTU in *Papa* samples; same was true for Solibacteraceae and mostly for Chthoniobacteraceae, which shared only one OTU in common.

Of those OTUs populated in both cultivars, some show differences in abundance or selectivity between bulk soil samples and root samples. For example, OTU 831386 (Syntrophobacteraceae) was much more strongly represented across all *Papa 'ele 'ele*

samples compared to *Hanakapi'ai*, but more importantly it was de-selected by *Papa 'ele 'ele* root samples (PS). We also noted that de-selectivity of family Mycobacteriaceae was strongly demonstrated in all OTUs of *Hanakapi'ai* root samples including the shared OTU 13034, which was only moderately abundant in all *Papa 'ele 'ele* samples.

| <i>Hanakapi'ai</i> # | <i>Hanakapi'ai</i> Bacteria | <i>Papa 'ele 'ele</i> # | <i>Papa 'ele 'ele</i> Bacteria |
|----------------------|-----------------------------|-------------------------|--------------------------------|
| 16 | Nitrosomonadaceae | 34 | Streptomycetaceae |
| 19 | Koribacteraceae | 35 | Saprospiraceae |
| 22 | Haliangiaceae | 36 | Rhizobiaceae |
| 26 | Conexibacteraceae | 37 | Pirellulaceae |
| 30 | Burkholderiaceae | 38 | Micrococcaceae |
| 32 | Acidobacteriaceae | 39 | Methylophilaceae |
| 33 | Acetobacteraceae | 40 | Erythrobacteraceae |
| | | 41 | Alcaligenaceae |

Table 11. HS/HC and PS/PC differences in bacterial family heatmaps.

Lists the 7 differing families found in *Hanakapi'ai* samples compared to 8 differing families found in *Papa 'ele 'ele* samples of the top 150 OTUs for each cultivar. Numerical label and Family identification are the same as for the original heatmaps annotations.

| Bacteria Family | <i>Hanakapi'ai</i> OTU | <i>Papa 'ele 'ele</i> OTU |
|----------------------|---|---|
| Xanthomonadaceae | 34580, 67341, 81089, 81358, 184325, 707290, 745642, 1118758, 2195500, 4343457 | 4337890 |
| Sphingomonadaceae | 243129, 1108062, 4447334 | 150689, 4258048, 4324733, 4344371, 4412910 |
| Sphingobacteriaceae | 357684 | 544883 |
| Solirubrobacteraceae | No OTUs of difference | 240137, 1012112 |
| Solibacteraceae | 204875, 224203, 666038, 812730, 3292593, 4298720, 4359889, 4361561 | 4397941 |
| Sinobacteraceae | 100307, 252012, 685739, 1125638 | No OTUs of difference |
| Rhodospirillaceae | 302726, 1108831, 4298129 | 240866, 250348, 262768, 4298215 |
| Pseudonocardiaceae | 4351616 | 918840 |
| Pseudomonadaceae | 270842 | 544313, 4451011 |
| Phyllobacteriaceae | No OTUs of difference | 513360, 795096 |
| Nocardiodaceae | No OTUs of difference | 305897, 888357 |
| Nitrospiraceae | 1491 | 4360284 |
| Mycobacteriaceae | No OTUs of difference | 13120, 354851, 736813, 4390723 |
| Micromonosporaceae | 112244, 214718, 248468, 249068 | 250989, 564025 |
| Hyphomonadaceae | 4468329, 4473304 | 4290381 |
| Hyphomicrobiaceae | 134163, 235789, 545247, 661494, 848909, 970359, 1144000, 4326152, 4337748 | 144675, 171619, 210344, 214791, 222792, 235789, 332595, 822874, 2727517, 3486915, 4101333, 4327829, 4354635, 4374806, 4380522, 4433035, 4477340 |
| Gaiellaceae | 1094433, 1122633, 4310013 | 244063, 247331, 257531, 311035, 551344, 561452, 642522, 938901, 3801733, 3843951 |
| Comamonadaceae | 4453710 | 153084, 644798, 818450, 819400, 1148345, 1673321, 4425152 |
| Chthoniobacteraceae | 207973, 224834, 240491, 1109646, 4310127 | 4350694 |
| Chitinophagaceae | 216350, 632692, 1110791 | 240838, 1133464 |
| Bradyrhizobiaceae | 4377104 | No OTUs of difference |

Table 12. HS/HC and PS/PC differences in OTUs representing the same bacterial family.

List of commonly occurring bacterial families on heatmaps in the top 150 OTUs for each cultivar represented by differing OTUs. Families containing the same OTUs as the other cultivar and showing no differences were indicated as no OTUs of difference.

3.5. Discussion

Direct influence of microbiome upon secondary metabolism has been demonstrated in plants of the *Cannabis sativa* family. An anecdotal example of this is the 1970's trend among illicit marijuana producers to allow marijuana to develop an associated fungal bloom before recreational use. The influence of the fungal pathogen here being to cause a dramatic enhancement of Δ^9 -tetrahydrocannabinol (THC) levels, presumably because THC is involved in plant anti-microbial defence responses. A second, more systematic study [89] examined soil determinants and cultivar specificity of *Cannabis* microbiomes. In this study, soil was the major driver of microbial community composition (biodiversity), but community structure (relative abundance of different microbes) was determined by the plant in the experiment. Cannabinoid diversity and composition was significantly correlated to the structure of the endorhizal communities, but this could not be dissociated from soil chemistry.

So little is known about the microbiome of medicinal plants, as previous research has been focused on agriculture for increasing survival and ultimately food yield. This exploratory study was designed to determine microbiome composition, potential differences in composition by 'Awa cultivar, and any differences as a result of traditional washing methodologies. Though differences in moisture content, seasons, time day and so many other factors can contribute to differences in bacterial composition, as with other soil microbiome studies, this is a snap shot in time of these 'Awa cultivars and still provides a wealth of information. Additionally, our results strongly suggest further studies are warranted in order for us to learn more about the importance of the kava microbiome as well as to determine why the similarities and differences that we discovered occurred; and more importantly, if and how that affects the secondary metabolism or contributes to the overall affect that 'Awa has on the user. One area that needs to be assessed across all studies of this type is the best way to process the samples. Since these replicates are taken from a larger sample, it is quite possible that the inconsistency seen in HC.2 and HS.2 are the result of 'hot' or 'cold' spots that can occur in soil sampling as a result of clumps, rocks and other debris that results in great differences in the overall shape and size of the particles within the same sample mass from which the DNA extraction took place. Consequently, results for relative abundance

run the gamut from higher to lower than other samples. Environmental studies of semi-volatile chemical pollutants has reported that depending upon the composition of the soil, the mass selected for testing can significantly under- or over-represent the concentrations of target compounds; new test methodologies now involve the use of micro-incremental sampling that requires pre-processing of samples into similar particle size before increments are systemically selected to provide the required mass of analytical testing. We wonder if a similar approach to microbiome studies should be employed to better determine the composition of bacterial communities, especially for bulk soil samples. We also acknowledge that OTUs may or may not represent a different species as organisms within the same species can differ genetically in the 16S region V4 rDNA region but at the same time we can discount the possibility that they are different organisms.

Previous studies of soil bacteria have shown that the majority of soil microbial communities are dominated by Proteobacteria and mostly contain these other phyla: Actinobacteria, Acidobacteria, Planctomycetes, Verrucomicrobia, Cytophagales, Bacteroidetes, and Firmicutes [109, 110]. For the most part, taxonomy and richness of our samples had compositions similar to these with the exception of Cytophagales not being present and the addition of small percentages of Chloroflexi and Gemmatimonadetes. The importance of this has not been determined as little is known of the soil microbiome composition in Hawai'i, with the majority of soil studies focusing on soil type. Despite these similarities, distinct differences in phylum composition and abundance were noted between the two cultivars: *Papa 'ele 'ele* (PC/PS) and *Hanakapi'ai* (HC/HS) in both bulk soil and roots samples when compared to the washed and unwashed roots of *Papa kea* (RC/RD). Washed and unwashed samples were composed of much higher percentages of Proteobacteria (~80%) while most other phyla groups were reduced by at least half compared to *Papa 'ele 'ele* and *Hanakapi'ai* samples with the exception of Firmicutes. Firmicutes composition remained similar in washed samples to percentages seen in bulk soil and roots samples for both 'Awa cultivars and interestingly increased in composition percentage in unwashed samples. Most likely, Firmicutes selectivity by root systems is inherent in their ability to suppress certain root rotting fungi [110].

With the criticism of the handling of 'Awa in the commercial setting and the question of how that contributed to the toxicity issues previously associated with the nutraceutical products, we wanted to look at how washing affects the microbiome on the roots. One area of significant change between the *Hanakapi'ai* and *Papa 'ele 'ele* samples and the *Papa kea* washed/unwashed group was the higher proportions of Proteobacteria and significant reduction of phyla from bulk soil and rhizosphere compared to the endosphere of roots is commonly seen for groups such as Acidobacteria, Planctomycetes and Gemmatimonadetes [111], thus in washed samples the near elimination of Gemmatimonadetes is consistent; however, unwashed roots still contained some rhizosphere soil yet their composition was nearly identical to washed samples whereas we expected a little more diversity in these epiphytes leaning more towards the composition in root samples of *Hanakapi'ai* and *Papa 'ele 'ele*. What was more interesting was the affect root treatment had on the microbiome. Significant separation of OTUs between washed (RC) and unwashed (RD) *Papa kea* samples were demonstrated with selectivity of certain taxa in the family Enterobacteriaceae being greater in washed samples, while possibly more important, deselecting for some potentially pathogenic species as demonstrated by the loss of Firmicutes: Bacilli in washed samples. However, due to the large presence of Enterobacteriaceae on both washed and unwashed roots, it is likely the result of contamination as roots harvested and process in non-sterile conditions and by hand. Many traditional practitioners have believed for sometime that 'Awa has probiotic benefits and has been given medicinally to treat gastrointestinal issues (personal communication). Additionally, some users of 'Awa have anecdotally reported stomach upset when large amounts of the beverage have been ingest at one sitting, which is a common issue for other types of probiotic treatments. Consequently the presence of Enterobacteriaceae bacteria helps to support both observations; however, near elimination of these families associated with probiotic bacteria suggests that probiotic benefits are from another source, such as how the beverage is made (i.e. reusing and nonwashing of the kava bowl and 'apu).

Phylum level heatmap showed that Actinobacteria and Acidobacteria were more abundant in washed samples then unwashed samples while the Family level heatmap showed the greatest selectivity for Enterobacteriaceae followed second by

Pseudomonadaceae. Certain OTUs were greater in abundance in washed samples than unwashed samples and included bacteria in the families Xanthomonadaceae, specifically *Stenotrophomonas geniculata*, which has been implicated in the degradation of certain terpenoids compounds [112] and may be important in concentrations of kava lactones in roots, since terpenoid compounds are precursors to lactones. On the other hand, some Enterobacteriaceae and *Pseudomonas* bacteria were decreased in washed samples with a significant decrease in an OTU identified as *P. viridiflava*, though not surprising that *P. viridiflava* is reduced on washed roots as it is a known phyto-pathogen that causes necrosis in the leaves, stems as well as root rot in food crops [113, 114]. Though not a probiotic, *P. viridiflava* presence is important to growers of 'Awa in that mild temperatures, frequent rain and relative high humidity are optimal conditions for the spread of this plant pathogen [113]. In general, *Pseudomonas* are found ubiquitously in the environment and contains the well-studied opportunistic pathogen *Pseudomonas aeruginosa* (Schroeter) found as part of the normal enteric flora as well as in biofilm formation [115]. More importantly, some *Pseudomonas* have also been studied for their bioconversion of terpenes into a wide variety of products from chemicals including those used in agriculture, antibiotics, antioxidants and anti-carcinogens [116]. Interestingly, kava has been studied for its potential as a chemopreventive in lung and bladder cancers in terms of the chalcones such as Flavokawain A, B and C and as fractions of the ethanolic extract [9, 10, 45, 117, 118], hence further studies should seek to find if there is any link between *Pseudomonas* conversion of certain root chemicals and previously report anti-carcinogenic effects.

With some washed samples showing an increase and other showing a decrease of bacteria in these genera, this suggests that 'Awa roots may select for specific bacteria; although the advantage or disadvantage of that is unknown at this time. More importantly, despite the differences between washed and unwashed roots, overall traditional washing does not appear to remove most of the bacteria, which may suggest that this method of washing exposes the bacteria found in the endosphere and that these endophytes may be selected for by 'Awa exudates. Studies in other plants have shown that the microbiome of plants is strongly influenced by rhizodeposition of plant exudates, which can contain sugars, organic acids and a variety of other compounds from hormones,

antimicrobials, amino and fatty acids, as well as sloughed dead cells and mucilage, and these chemical factors are known to vary significantly between species, cultivars and plant age [110].

Traditional practitioners of 'Awa use and prescribe different strains depending upon the condition or reason; for example, *Hanakapi'ai* is often prescribed prophetic sleep where answers are sought while *Papa 'ele 'ele* is used to quiet the mind and provide a more restful sleep [50]. Many cultivars not only have a different outside appearance, but also differ in their chemical composition and concentration of major components like the kava lactones [119, 120], so we suspected some variation in the microbiome of our two cultivars, *Hanakapi'ai* and *Papa 'ele 'ele*. Instead, we found significant differences between the two cultivars as seen in both the phylum and family level heatmaps and even between shared OTUs. Both cultivars contained certain additional bacterial families that were consistently strong in abundance across all samples, most of which differed in OTUs. *Hanakapi'ai* and *Papa 'ele 'ele* samples also demonstrated possible selectivity and de-selectivity of certain OTUs and families by root samples as indicated by higher abundances in root samples compared to bulk soil samples and root samples with *Papa 'ele 'ele* also suggesting a higher selectivity diversity with at least 29 OTUs with some level of selectivity compared to *Hanakapi'ai* with only 17 OTUs. Both cultivars also demonstrated the possibility of de-selectivity of certain OTUs and families as indicated by higher abundances in bulk soil samples compared to root samples. *Hanakapi'ai* contained three other families with strong to moderate trend for de-selectivity: Acetobacteraceae, Hyphomonadaceae and Xanthomonadaceae; however, *Papa 'ele 'ele* de-selected for six different families: Nocardiodaceae, Phyllobacteriaceae, Rhodospirillaceae, Saprospiraceae, Sphingomonadaceae and Syntrophobacteraceae. This suggests that either *Papa 'ele 'ele* may be more inclined to lower the populations of some bacteria in favour of other beneficial bacteria or that this de-selectivity is the reason for *Papa 'ele 'ele* samples appearing to be more diverse.

3.6. Acknowledgments

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CHAPTER 4

4. CONCLUSIONS AND DISCUSSION

Primarily, kava research has focused on the kava lactones and their effect on the CNS and GABA receptors for its relaxation and anti-anxiety benefits for both the cultural/social beverage as well as the nutraceutical supplements. Results from chapter 2 showed that kava has complex cellular responses outside of the CNS/GABA, and that non-kava lactone components initiate other cellular responses, especially those associated with calcium ion channels, TRP and I_{CRAC} . These results indicate that a strong and important connection between non-kava lactones and some of the documented physiological as well as potential pathophysiological responses. These secondary metabolome products are understudied, not only in kava, but also in other potential medicinal plants, to include *C. sativa*, which is used here as an example. Cannabis contains more than 400 known bioactive molecules, but knowledge of their mechanistic action is extremely limited as the focus has been primarily on the cannabinoid compounds [48, 49].

In the case of kava, the focus has been on the kava lactones due to their high concentrations (96%) in extracts [36]; however, the remaining concentration contains a plethora of other kava components, which include the dihydrochalcones (flavokawains A, B and C), flavanones (5,7-dimethoxyflavanone, 5,7-(OH)₂-4-one- 6,8-dimethylflavanone, 5-hydroxy-4',7-dimethoxyflavanone), chalcones (pinostrobin chalcone), and tentatively identified compounds in the classifications of phenolics, and fatty acids (8,11-octadecadienoic acid-methyl ester) as well as cinnamic acid bornyl ester [8]. The latter, cinnamic acid, is a known transient receptor potential (TRP) channel activator, specifically TRPA1 in mast cells, which is associated with contact dermatitis [39, 40] and thus the potential receptor and associated molecule that may be responsible for the kava dermopathy. Another TRP channel, TRPV, are stimulated by chemicals such as capsaicin and are responsible for the burning sensation felt from hot peppers [121], with this suggesting that a related kava component may be responsible for the tingling sensation associated with the kava root/beverage. Other TRP channels are potential targets of kava secondary metabolites and based on their locations and non-responses and may also explain some of the efficacy and possible toxicity, specifically, TRPP, found as membrane bound proteins on tissues such as the kidneys, may be a link to the kidney and liver toxicity; TRPA channels, associated with apoptosis [121], may explain cellular tissue damage as well as the

potential mechanism for anti-carcinogenic properties briefly mentioned in the introduction. Chapter 2 results strongly suggest that the kava-lactone-centric viewpoint, which is not to say that these responses are not equally important, under estimates the impact of other cellular responses on efficacy and potential toxicity.

With the emerging evidence that kava has a wide-range of secondary metabolites, but also based on the results of chapter 2, contain a number of bio-active molecules that induce non-CNS/GABA targets, it is important to look at factors that impact the production kava phytochemicals. One such important factor is the microbiome of the plant and surrounding soil; in fact, studies on *C. sativa* have shown that the microbiome influences changes in metabolic products to include THC levels. Combined with the traditional practitioner knowledge that different growing locations/conditions influence the properties of the kava cultivar, which results in how they use and prescribe the strains, suggests that the microbiome may play an important role in the differences in metabolite production. In order to dive into these concepts, a general study into the microbiome of the plant and surrounding soil needed to be addressed, and here, in chapter 3, we undertook the challenge of describing the first kava microbiome. Compared to other published soil microbiome results, no discernable revelations were made outside of slight differences in soil phylum composition; however, prior to this, studies on Hawai'ian soil have focused on physical and chemical characterizations for agricultural purposes.

On the other hand, comparisons between *Hanakapi'ai* and *Papa 'ele 'ele* cultivars did reveal significant differences in their microbiomes that may help to later explain the important distinctions in the properties of these two cultivars. For starters, family level comparisons showed an overall difference in abundance and composition with *Papa 'ele 'ele* generally lower in abundance values but slightly more diverse than *Hanakapi'ai* samples. Furthermore, many more OTUs representing these families were more strongly represented in *Papa 'ele 'ele* bulk control soil and root samples suggesting that this cultivar is either less selective of bacterial species, or possibly, the plant is less influenced by the microbiome. Also, *Hanakapi'ai* and *Papa 'ele 'ele* were represented by some differing families in the top 150, seven and eight different families respectively. Interestingly, even within shared families, each cultivar was either represented by different OTUs, or in some cases, in a higher number of OTUs affecting the percentages in which these families were represented in each cultivar. Overall, at the OTU level, despite a general similarity in sharing most family categories, *Hanakapi'ai* and *Papa 'ele 'ele*

share few species with each other. The importance of this cannot be determined from what we currently have discovered in chapter 3; nevertheless, these results strongly suggest enough differences to have a potential effect on important pathways associated with the generation of secondary metabolites and even the concentrations of the kava lactones.

In chapter 3, at the request of the kava community, we also investigated the affect that washing techniques have on kava roots of *Papa kea*, as well as, the potential that kava is probiotic. Unwashed roots (with soil brushed off) as well as power washed roots did show significantly lower phylum level richness and concentrations compared to *Hanakapi'ai* and *Papa 'ele 'ele* root samples, but washing did not removed most or even all of the microbiome suggesting that endophytes within the root interior are exposed. Results in chapter 3 also indicated that both washed and unwashed *Papa kea* samples did associate with known-gut bacterial families with at least one OTU in the family Enterobacteriaceae higher in abundance on washed samples. However, due to the large presence of this family (54%), it may be the result of contamination since it is standard for kava roots to be processed by hand and under non-sterile conditions. Despite this, it does appear that at minimum, washing does remove most potentially pathogenic species. In terms of probiotic impact, results did show that families associated with probiotic species do associate with roots, but washing nearly eliminates these bacteria; as a result, any probiotic benefits attributed to kava must be the result of how the drink is later prepared such as re-using and not washing of the kava bowl and the 'apu. Of potential agricultural importance, *Papa kea* results did show that a few plant pathogenic species do associate with the roots, most notably *P. viridiflava*, which causes root, stem and leaf necrosis, and genus *Erwinia*, which is associated with blight. This is important to kava growers since new plants are developed from the stocks of previous generations, so care must be taken to avoid spreading and contaminating the next generation of kava plants and soil as well as avoiding soils or treating soils for these pathogens prior to planting.

On a final note, the success of this project could not have been completed without the support and sharing of the kava community. It is imperative that the scientific community embraces and values the knowledge of traditional practitioners for the wealth of understanding through their generations of working with and learning from the kava plant. To attempt to do so without them places the scientific community in a knowledge gap that will continue to place the public at risk. This must be applied to other natural product plants as well, as Western medicinal

industrial complex as well as the consumer continue to search for alternative treatments to combat the array of side effects and perceived greed associated with pharmaceutical drugs and industry. Working together as partners, will create more understand about these plants as well as build appreciation for them, for not only there complexity, but there importance in traditional society, and together, these two communities will able to solve problems and health concerns on both sides of the spectrum.

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APPENDIX 1



Review

Contemporary Pacific and Western perspectives on `awa (*Piper methysticum*) toxicology



Angelique F. Showman^{a,c}, Jonathan D. Baker^b, Christina Linares^a, Chrystie K. Naeole^a, Robert Borris^d, Edward Johnston^f, Jerry Konanui^f, Helen Turner^{a,c,e,*}

^a Laboratory of Immunology and Signal Transduction, Division of Natural Sciences and Mathematics, Chaminade University, Honolulu, HI, United States

^b Department of Anthropology, University of Hawai`i, Honolulu, HI, United States

^c Graduate Program in Molecular Biosciences and Bioengineering, University of Hawai`i at Mānoa, United States

^d Daniel K. Inouye College of Pharmacy, University of Hawai`i at Hilo, Hilo, HI, United States

^e Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawai`i, Honolulu, HI, United States

^f Association for Hawaiian `Awa, Hilo, HI, United States

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ABSTRACT

In 2010, a National Science Foundation project in Hawai`i assembled a collaboration of Pacific indigenous scientists, Hawaiian cultural practitioners and scientists trained in Western pharmacology. The objective of the collaborative project was to study Kava, a culturally significant Pacific beverage, and to address and ultimately transcend, long-standing barriers to communication and collaboration between these groups. Kava is a product of the `awa plant (*Piper methysticum*) that has been used ceremonially and medicinally throughout the history of Pacific Island cultures, and is now in widespread recreational and nutraceutical use in the US. This project, culminating in 2015, has enriched the participants, led to published work that integrates cultural and Western pharmacologic perspectives and established a paradigm for collaboration. This review paper integrates cultural and Western perspectives on efficacy, toxicity and the future cultural and commercial significance of `awa in the Pacific. Here we present a detailed review of traditional and non-traditional kava usage, medicinal efficacy and potential toxicological concerns. Recent mechanistic data on physiological action and potential pathological reactions are evaluated and interpreted.

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* Corresponding author at: Chaminade University, 3140 Waiālae Ave, WSC116, Honolulu, HI 96816, United States. Tel.: +1 808 440 4204.

E-mail address: hturner@chaminade.edu (H. Turner).

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1. Background—Kava—historical and geographical perspectives

Piper methysticum Forst. f., meaning, “intoxicating pepper,” is a shrub-like plant known predominantly as kava, or `awa to the Native Hawaiians. It is native to Oceania, growing throughout Polynesia, Melanesia, and Micronesia [1]. The plant was domesticated ~3000 years ago in Vanuatu, and spread throughout Oceania via Austronesian colonists [2]. In the Native Hawaiian culture, `awa is described as coming to Hawai`i with the akua (gods) Kāne and Kanaloa. Kāne is believed to have made water appear to nurture the `awa crop.

Traditionally, the consumption of kava as a beverage was sacred in Pacific cultures. Indeed, in the words of Mary Kawena `ulaokalanihi`iakaikapoliopolekawahine`aihonua`ināle ilehuaapele Pukui “.....`awa was the food of the gods.....no religious ritual was complete without it” [3]. Offerings of `awa were made to protect the health of the Hawaiian people, in rites of passage, to lift tabus and to both facilitate consensus-building and prepare for war or battle. Margaret Titcomb [4] summarized usages of `awa: “The `Awa custom is of interest in Hawai`i because it was a sacred drink of importance in many phases of Hawaiian life. ... Its effect is to relax mind and body Medical kahunas (learned men) had many uses for it.....It was essential on occasions of hospitality and feasting, and as the drink of pleasure of the chiefs”. A Hawaiian mele illustrating these usages is shown at the left.

*He `awaawa no nā kāne
A me nā wahine o ka lani He `awaawa no nā kāne
Me nā wahine o ka lua. Pēlā aku, pēlā mai,
E mū ka waha, e,
E holoi i ka lima.
Elieli kapu, elieli noa, Noa ke kapu, noa ka hele,
Noa kānawaawai a ke akua*

*`AwaAwa for the men,
And women of the heavens,
`AwaAwa for the men,
And for the women of the pit.
Thus it was, thus it is,
Silence the mouth,
Wash the hands.
Sacred is the taboo, sacred is the freeing,
The taboo is lifted and one can go,
The law is lifted by the gods*

Various parts and preparations of `awa were used medicinally in Pacific cultures. From their earliest contact with Pacific

islanders, Europeans were therefore interested in kava as a medicine, first as a treatment for venereal diseases [5], and later as a sedative and treatment for anxiety [2]. A major boom in kava popularity occurred in the 1990s linked to both health-related and recreational usage for non-Pacific audiences. The most recent incarnation of the kava story is as a nutraceutical, formulated as pills and liquid extracts, as an analog to anti-anxiety drugs. Products are standardized to a specified concentration of kavalactones, which have been extracted from kava plant material with alcohol, acetone, or water.

2. Contemporary exposures to Kava

In the contemporary Pacific, people still drink kava. The drink is still prepared in a semi-traditional manner as a water extract served from a common bowl into smaller drinking cups (often coconut shells). The drinking protocols and associated social meanings continue to evolve. There is an awareness of the traditions associated with kava, even if little of this knowledge is incorporated into the actual way the beverage is consumed. The purpose of contemporary consumption is largely consonant with less formal consumption of earlier times, but the frequency of consumption, amount consumed, and social context of kava drinking also reflect modern shifts in perspective and social relations. Current exposure is in some cases significantly different from that in the past. As such, a review of kava's safety should examine these shifting and nuanced social dynamics, rather than reiterating past dichotomies of traditional/nontraditional consumption [6].

Contemporary kava use presents two distinct patterns of consumption. Kava drinking is social, involving relatively high doses, and the dosage is not strictly controlled or limited. Kava nutraceutical consumption is of a fixed recommended daily dose for the goal of treating a specific medical condition; it is personal rather than social. Traditionally, kava is mixed with water, is not extracted with another solvent, is strained by hand, and is prepared as a social drink. By contrast, nontraditional nutraceutical forms of kava are solvent-extracted (alcohol or acetone), usually as part of a commercial process, and not consumed socially. A full description of traditional kava drinking and nontraditional consumption is beyond the scope of this paper [2,6], but there are some salient points for comparison when thinking about dosage, effects, and possible risk from these different consumption practices. First, the amount of kava consumed by drinkers is significantly higher than that consumed by those taking supplements. Kava drinkers will normally consume several coconut shells of kava beverage in a typical drinking session. On average, each shell contains as much more than the recommended daily dose of kavalactones used in supplement form for treating anxiety (~200 mg). A

night's dose of kavalactones from drinking kava (5–10 shells) could easily be in the range of 1.0–1.5 g. It should be remembered that these two forms are not identical, as they are prepared by substantially different techniques, and using different solvents. Second, kava drinking is a social activity, whereas supplement consumption is a personal activity with no inherent social dimension. Third, kava beverage is not a standardized product, whereas nutraceuticals supposedly are.

It is difficult to accurately determine the number of people who are consuming kava, the amount of kava they are taking, and the frequency with which they use it [7]. Global use of kava supplements is certainly substantially lower than it was in 2001, prior to bans instituted by several countries due to concerns about liver toxicity [8–15]. However supplement use in the US continues; consumption of kava as a social beverage seems to be increasing [16–23]. Data on the amount of kava produced and exported are not accurate, sales figures for kava products are not widely available, and it is difficult to estimate number of users since both the production and consumption sides of the commodity chain are fragmented.

Kava is grown in more than 6 different island nations in the Pacific and in the wider Pacific Islander diaspora. It is consumed locally and exported to the United States for manufacture into nutraceuticals. Fiji, Vanuatu, Samoa, and Tonga are primary kava-exporting countries [24]. Export statistics from the producer nations give a partial glimpse of consumption, but they are not widely available or reliable. The decentralized, minimally regulated nature of kava's commodity chain contributes to this uncertainty. With respect to supplement use, several companies produce kava supplements (and other products such as kava skin creams), primarily for the US market. There is the potential for this market to dramatically increase, following a 2014 court decision in Germany that overturned the ban on kava products in that country. Changes such as this to the regulatory frameworks in which kava is embedded could quickly affect the availability of these products.

Kava consumption in the US has expanded through supplement availability and most recently through the proliferation in kava bars. In the Pacific, there have also been changes in the pattern of kava consumption. Migration within the region has brought kava drinking to places where it was not previously a tradition (e.g., Kiribati, New Caledonia, the Solomon Islands, and New Zealand; see [6,25]). In addition, changing social practices in societies for which kava drinking is a tradition may be leading to increased consumption. For example, more women are drinking kava in the Pacific than in previous decades. The aggregate result is more demand for kava for general consumption, and more kava consumed in those drinking sessions, as well as an increase in growing kava [26]. In light of these points, a review of the potential toxicity of kava is timely.

3. Kava pharmacology and targets of action

The known active ingredients in kava are the kavalactones. Eighteen of these have been identified, but only six of them: methysticin, dihydromethysticin, kawain, dihydrokawain, desmethoxyyangonin and yangonin, have been the focus of kava studies as they make up 96% of organic extracts [27]. See Fig. 1 for structures of major kava components. However, kava extractions contain a variety of other non-lactone compounds, which may be responsible for the pharmacological

benefits and potential toxicity [27,28]. The activity of kava may be the result of one of these non-lactone compounds or a synergy of several or all components found in kava. In fact, studies on RBL2H3 mast cells (Rat Basophilic Leukemia subtype 2H3, ATCC CRL-2256), showed that traditional aqueous kava extracts elicited strong calcium responses not seen in individual or combined purified kava lactones, specifically methysticin, dihydromethysticin and kawain [29]. Furthermore, traditional aqueous kava extracts demonstrated mast cell degranulation whereas purified lactones did not [29].

A focus on kavalactones may neglect other important compounds in kava that can direct cellular responses. Additional kava components include the dihydrochalcones (flavokawains A, B and C), 5,7-dimethoxyflavanone, cinnamic acid bornyl ester as well as tentatively identified compounds classified as phenolics, flavanones, fatty acids and a chalcone, specifically 2,5,8-trimethyl-1-naphthol, 5-methyl-1-phenylhexene-3-yn-5-ol, 8,11-octadecadienoic acid-methyl ester, 5,7-(OH)₂-4'-one-6,8-dimethylflavanone, 7-dimethoxyflavanone-5-hydroxy-4' and pinostrobin chalcone [28]. Cinnamic acid has been shown to activate the mast cell calcium channel TRPA1 and has been associated with contact dermatitis [30–32] while the pinostrobin chalcone can act as a stimulatory or inhibitory molecule on mast cells [33]. Equally important, the type of extraction solvent used has a marked effect on the ratios of these compounds as well as the kavalactones, with some compounds not being extracted at all [28].

Though studies have focused on the kavalactones being the primary components of kava extractions, correctly since they have demonstrated a mechanistic connection to gamma amino butyric acid (GABA) receptors, the chemical complexity of kava extracts suggests that a kavalactone-centric approach may (1) underestimate the complexity of and (2) not provide a mechanism for some of the non-GABA based medicinal effects.

4. Kava side effects

Controversy between the approaches of Western kava use and its traditional counterpart arises when considering its medicinal role and the possible pathological side effects on human physiology [34,35]. The pathophysiological effects of kava include muscle degradation, kava dermopathy presented as scaly skin rashes, urticaria, sebotropic eruption, meningism, depression/suicidal tendencies and hepatotoxicity [11,13,36–44]. Amongst traditional practitioners, chronic use of kava has been associated with exfoliating dermopathy [13,36,37,40] that is acknowledged as common. However, long-term health effects such as hepatotoxicity and carcinogenic activity are generally and historically unknown in these same Pacific cultures. Whether this is the result of underdiagnosis, generally poor health surveillance or genuine protection against adverse affects (via genetics, usage patterns or preparative methods) is a crux of current debate. Conversely, the adverse events reported in the Western scientific literature may reflect preparations, material origins and co-morbidities with alcohol, other supplements or prescription drugs that are not dominant in Pacific cultures.

Concerns about possible toxic effects of kava arose in Western countries when reports from Germany, Switzerland and the United States allegedly linked the use of kava containing products with liver failure [45,46]. From 1999 to 2002, a

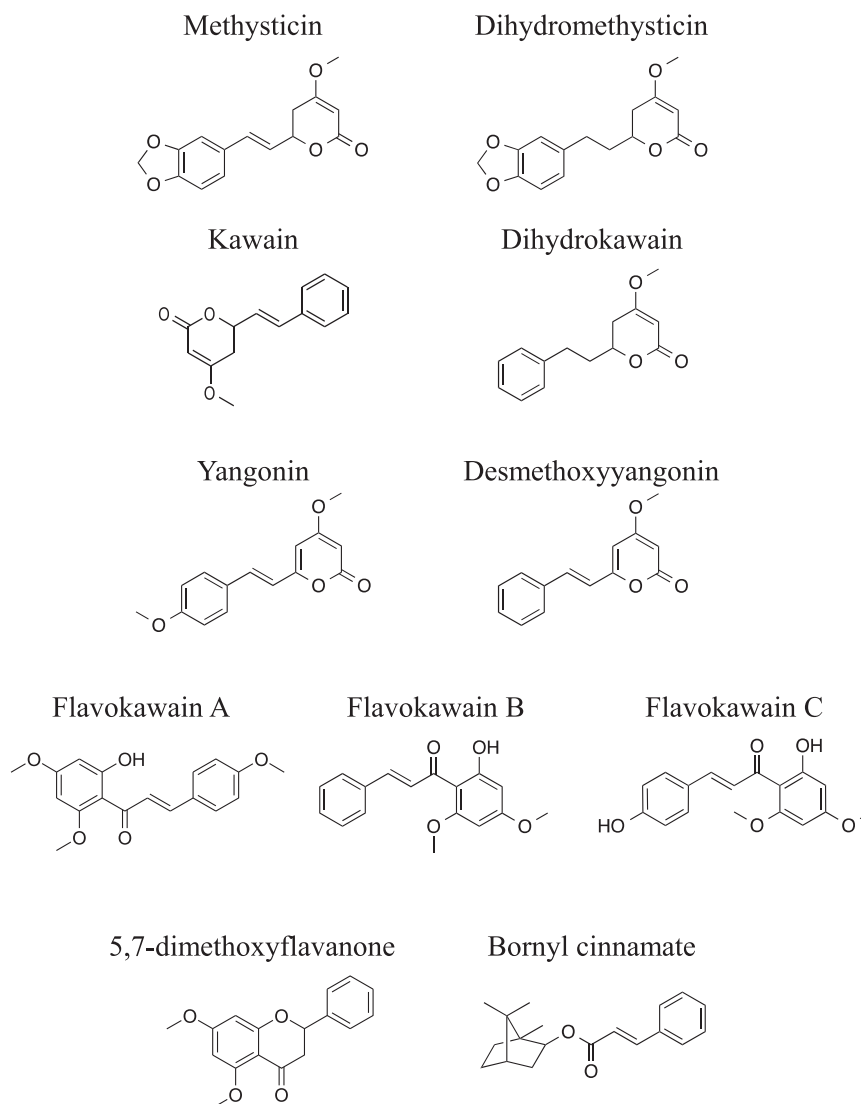


Fig. 1. Kava constituents discussed in the text.

total of 10 patients; six in Germany, two in Switzerland, and two in the U.S. required liver transplants after using products containing kavalactones at doses ranging from 60 mg to 240 mg for as little as 8 weeks to as long as 12 months [9,11,13,41,47,48]. Both patients in the U.S. reported taking kava supplements in capsule form while most European patients reported kava prepared by extraction with either acetone or ethanol [41]. CDC advisories were released in 2002 and 2003 [15,41]. Due to potential toxicity, kava was banned in 2002 by the German Federal Institute for Drugs and Medical Devices (BfArM) and the British Parliament followed suit banning the sale of all products containing kava in 2003 [8–13,49]. However, on June 10, 2014, the German Administrative Court overturned the 2002 ban reinstating the regulatory requirements of 2001. This court stated that risk from kava exposure has not been clearly demonstrated nor appears unusually high, an opinion presumably driven by the very small number of cases of reported toxicity ($n \sim 3$) with even a certain degree of causality linked to

kava in a global kava-consuming community that may number in the millions of doses consumed daily.

As we review below, Western science has reported inconsistent information with some studies showing human kava use with hepatotoxicity ranging from cirrhosis, hepatitis and even liver failure [9,11,13,41,47] and some which do not [50–52]. A range of side effects and adverse effect outside the liver are suggested by human cases and animal studies [34,35]. Animal studies demonstrate carcinogenicity of kava, and yet chemopreventive actions, creating a confusing picture for consumers and health professionals. One question which is little addressed in the literature concerns whether under-reporting or diagnosis of health conditions contributes to the perception that kava is not dangerous in the Pacific. This is a clear critique of the work of Steiner which is oft-quoted as an epidemiological basis for the cancer-preventive effects of kava, and by extension as a logic that kava in the Pacific ‘does no harm’ [53]. The Steiner study draws a correlation between kava use and ‘low’ cancer rates in Pacific

countries, but fails to address a multitude of confounding factors and alternate explanations, and the high potential for under-reporting/under-diagnosis of cancer rates in Pacific island societies with challenges in health care access and provisions such as pathology services. Absent more evidence, the Steiner study is an exemplar of the *cum hoc ergo propter hoc* (Lat., *with this, therefore because of this*) class of epidemiological fallacy where association cannot be used to infer causation.

Pacific practitioners address the apparent paradox (the toxicity associated with 'Western' use but not in indigenous cultures) through drawing attention to differences in extraction method, uninformed use of particular cultivars or ill-advised production of the kava drink from parts of the plant not in traditional use. This paradox, together with the explosion of relatively uncontrolled mixes and preparations of kava constituents in the nutraceutical market and a concomitant increasing deviation from traditional preparative methods, creates a need to: (1) review the depth and strength of current data on toxicity, (2) reconcile the apparent paradox between the Generally Regarded as Safe (GRAS) status of traditionally prepared beverages in Pacific cultures with studies and clinical experiences that suggest toxicity, and (3) develop recommendations informed by traditional practices that support safe exploitation of the potential medicinal and nutritional benefits of kava and its many bioactive components.

5. Recent progress in kava toxicology

Here, a range of studies is reviewed across *in vitro* and *in vivo* systems that examine the toxicity of kava and kava components in aqueous and organic preparations.

5.1. National Toxicology Program study

A comprehensive toxicology study in rodents was performed by the National Toxicology Program (NTP) and published in 2012. This was a 2-year kava gavage study in F344/N rats and B6C3F1 mice. The study revealed equivocal evidence of carcinogenic activity among male rats, and clear evidence of carcinogenic activity in male mice with some evidence of carcinogenic activity in female mice. In addition, kava extract in male and female rats resulted in an increase of tumor-like lesions in eyes, kidneys, liver, pancreas and rumen. Note that Equivocal Evidence is defined as marginal increases in neoplasms that may be chemical related, and Some Evidence is defined as a chemical-related increase of malignant, benign or a combination of neoplasms with a response in strength less than that defined under the NTP guidelines for Clear Evidence of carcinogenic activity [54].

The bulk powdered kava extract used in the NTP study (Cosmopolitan Trading Co.; Seattle, WA; Lot 9077SDK) was tested for purity, stability, organic constituents, and identity using various chromatographies. Methanol and aqueous extractions tested by HPLC/UV and LC/MS identified six kava lactones present in the powdered extract: methysticin, dihydro-methysticin, kavain, yangonin and desmethoxyyangonin. LC/MS tentatively identified seven additional compounds. Cadmium, lead, mercury, organochlorine and organophosphorous pesticide contaminants were below detectable limits. The bulk kava extract contained 1.4 ppb N-nitrosodimethylamine

(NDMA) and 31.2 ppb N-nitrosopyrrolidine [54], both of which can be hepatotoxic in rodents at 1 mg/kg levels [55].

A three-month study was designed to determine any additive toxic effects to kava exposure and determining best concentrations for a two-year study. Upon terminal sacrifice no gross lesions were observed but both liver weights were increased in all male rat groups and in at some female rat doses. Microscopic visualization showed hepatocellular hypertrophy in female groups. Male mice in some dose groups displayed centrilobular hypertrophy. Absolute liver weights were significantly increased and male rats at week 14 showed a decrease in alkaline phosphatase (ALP), alanine aminotransferase (ALT), and sorbitol dehydrogenase (SDH). Both male and female 2.0 g/kg rats showed a significant increase in cholestasis serum marker γ -glutamyltransferase (GGT); however, all other cholestasis markers were either decreased or unaffected [54].

In the two-year core study, lethargy and lack of muscle control occurred within the first four weeks of the study; these incidences appeared to decrease in the number of rats over time but reappeared periodically through the remainder of the study at this same dosage. Around the first year of the study, some rats experienced seizures. Hematology results in both male and female rats also showed a decrease in ALP, ALT and SDH and increases in cholestasis markers γ -glutamyltransferase and bile salts [54]. Unlike the 3-month study groups, 'statistically significant or biologically noteworthy' tumor and/or tumor-like lesions appeared in the two year study [54]. An increase in the prevalence of hepatocellular hypertrophy and instances of fatty change [54] were observed in liver [56]. Cystic degeneration and significant increases in multiple hepatocellular adenomas were observed. Malignant liver tumors significantly increased in male mice (in the form of hepatoblastomas) and increased in all kava-exposed female mice (in the form of hepatocellular carcinoma). Male and female mice displayed a significant increase in centrilobular hypertrophy and hepatocellular necrosis [54].

Other organs also showed changes after kava exposure. Significant increases in inflammation, formation of ulcers and increased growth in epithelial cells were seen in the forestomach. Increases in kidney damage, transitional epithelial hyperplasia of the renal pelvis, parathyroid gland and in the bone marrow hyperplasia and retinal degeneration were observed. Leydig cell and bilateral interstitial cell adenomas occurred and kava dose correlated with increase in severity of hyperplasia. Microarray data and immunohistochemistry examined any possible mechanisms for the liver toxicity seen in the animal studies. These tests showed that kava alters cytochrome P450 family of drug metabolizing enzymes (specifically CYP1A1) dose-dependently [54].

In summary, evidence from the NTP studies showed that kava exposure impacts liver function and is most likely dose-dependently and chronically toxic as demonstrated by the significant increases in GGT concentrations, hepatocellular hypertrophy and other histological observations in the three-month and two year studies as well as the effects seen on P450 liver enzymes. Liver toxicity did occur in both rats and mice. Despite the differences in tumorigenesis noted between the animal species, the conclusion of the two-year study stated that "there was equivocal evidence of carcinogenic activity in male F344/N rats based on marginal increases in the incidences of testicular interstitial cell adenoma", "clear evidence of carcinogenic activity of kava kava extract in male B6C3F1 mice based on

increased incidences of hepatoblastoma" and "some evidence of carcinogenic activity of kava extract in female B6C3F1 mice based on increased incidences of hepatocellular adenoma or carcinoma (combined)" [54].

5.2. Flavokawain B is a hepatotoxic constituent from kava root

Chalcone kava components have been linked to the hepatotoxic effects seen in kava consumption [51]. HepG2 hepatoma cells exposed to ethanolic extracts of the kavalactones shown to contain kawain, dihydrokawain, methysticin, dihydromethysticin, yangonin and desmethoxyyangonin separately via HPLC showed no toxicity at 150 μ M with the exception of yangonin demonstrating an LD₅₀ toxicity at 100 μ M; whereas, kava root extracts containing Flavokawains A, B and C (FKA, FKB, FKC) showed significant toxicity. FKB showed the highest *in vivo* toxicity with an LD₅₀ value of ~15 μ M, and inhibited NF κ B activity *in vitro*. These results suggest that, for the most part, the kavalactones are not the source of toxicity with only yangonin being weakly toxic [51]. FKB hepatotoxicity was confirmed *in vivo* when male ICR mice were fed 25 mg/kg body weight FKB for one week. FKB fed mice showed substantial liver damage (hepatocellular swelling, vesicles appearing in the cytoplasm, and inflammation in the periportal area). These results strongly suggest that FKB is hepatocellular toxin not only *in vitro*, but also *in vivo* [51].

These studies demonstrate that kava has potential toxicity in both *in vitro* and *in vivo* models, yet understanding of the mechanism involved in kava toxicity is unclear. Several mechanisms have been proposed that may explain not only the toxicity seen in the above models as well as in the few human cases, but also may explain why the toxicity is reserved to a small number of individuals and why this same toxicity is not observed in Polynesian populations.

6. Mechanisms of toxicity

6.1. Importance of the P450 enzymes and the effect of kava on CYP genes

Inhibition or other defect in CYP genes or direct inhibition of the P450 enzymes is of concern when ingesting any chemical. Approximately 1% of Polynesian and nearly 1% of Asian populations have CYP2D6 deficiency; whereas, approximately 6% of Western Europeans and up to 9% of Caucasians are CYP2D6 deficient [54]. Some studies have suggested that kava toxicity in humans is due to a deficiency in the CYP2D6 gene, responsible for coding the cytochrome P450 family of oxidase enzymes [57–59]. *In vitro* experiments have shown inhibition of several CYPs when exposed to kava extracts and kava alkaloids; it is believed that this inhibition increases the possibility of cytotoxicity due to drug interactions when both kava and other chemicals are consumed concurrently [58,60]. Methysticin analogs found in kava contain a methylenedioxyphenol group, which after metabolism, demonstrated inhibition of several P450 enzymes. Kava extracts, normalized to 100 μ M kavalactones with NADPH, severely inhibited CYP2C9, CYP2C19 and CYP3A4. Of the individual kavalactones, desmethoxyyangonin showed significant inhibition of CYP2C9 and CYP3A4. Methysticin showed inhibition of CYP2C9, CYP2D6 and CYP3A4; dihydromethysticin inhibited CYP2C9, CYP2C19, and CYP3A4. However, kawain

demonstrated no inhibition of these families of P450 enzymes [58,60]. Thus, inhibition of P450 enzymes by kava may be responsible for drug interactions and liver toxicity; and desmethoxyyangonin, methysticin and dihydromethysticin may be competitive inhibitors of P450 enzymes [58].

6.1.1. Influence of extraction method on kava toxicity

The bioavailability of kava components varies with extraction conditions [27,28]. Nutraceutical production of kava products favors organic extraction methods over the traditional water extraction, because they result in higher concentration of kavalactones than aqueous extraction [8]. Analyses comparing water-extracted kava to acetone, ethanol or methanol extractions have demonstrated differences in the kavalactone ratios and representation of polar compounds [27,28]. Despite these differences in extraction methods, it is still unclear if kavalactone overdose *per se* is the root cause of any toxicity. However, the elevated levels of kavalactones in organic extracts (especially those that are over-represented relative to traditional aqueous preparation) are often pointed to as a potential source of toxicity in organic/nutraceutical preparations. It should also be noted that carbon dioxide extraction is used in some nutraceutical preparations, but the chemical profile of extracts from this methodology is not published, and their relative efficacy/toxicity has not been studied comparatively in *in vitro* or *in vivo* systems.

6.1.2. Genetic influences on kava toxicity

CYP2C9, CYP2C19 and CYP2D6 are the most polymorphic CYP [61]. Drug metabolism is either classified as monogenic, or polygenic, or polymorphic (a monogenic trait that has two or more phenotypes and genotypes in a population) variations that result in individual differences in drug metabolism. Individuals are poor, extensive or ultrarapid metabolizers (PM, EM, UM). PMs have a deficiency in drug metabolizing enzymes, which leads them to increased risk of toxicity due to drug accumulation of both the active compound as well as any metabolites [62]. EMs and UMs, though able to quickly metabolize drugs have their own concerns in that therapeutic benefits of such compounds may require higher doses making determination of safe levels of any drug for an entire populous difficult to determine.

CYP2D6 (debrisoquine/sparteine hydroxylase) is believed to metabolize at least 25% of all common drugs [61]. The gene locus is polymorphic with at least 70 allelic variants. Variant D6 alleles (most notably D6*2, D6*4, D6*5, D6*10, D6*17 and D6*41) are responsible for poor, normal and extensive (ultra) metabolizer phenotypes as well as completely annulled activity [61]. Genetic differences in CYP2D6 genes between traditional-kava drinkers of Polynesian decent versus nutraceutical-kava users of non-Polynesian decent, have been hypothesized as a possible cause of the kava toxicity in a few of the previously reported cases [63]. In two cases of toxicity, the patients tested as CYP2D6-deficient [8,11]. Differences in ability to metabolize racemers of drugs may also link to CYP genotype. For example, CYP2C19 metabolism of S-mephenytoin EMs are identified as those able to completely hydroxylate the S-enantiomer with PMs showing a deficiency in the ability to undergo this reaction [62,64].

Differences in polymorphic CYP enzymes may affect the functionality or toxicology of Western medicines and nutraceutical alternatives. Moreover, the dominance of these genes in mixed ethnic individuals is not clearly understood, indicating

that the incidences of kava toxicity in these individuals may be higher than previously reported. For example, the incidence of debrisoquine (CYP2D6) PMs in the New Zealand Maori population consisting of mixed racial backgrounds primarily with Caucasian lineage has been reported at 5% and proguanil (CYP2C19) PMs at 7%, suggesting that mixed genetic background is extremely important in the drug metabolism [64]. Due to the origination of South Pacific Polynesians from Southeast Asia, both CYP2D6 and CYP2C19 polymorphisms were studied in unrelated South Pacific Polynesian volunteers and compared to the known polymorphisms in Asians and Caucasians [64]. Volunteer Cook Islanders, Niueans, Samoans and Tongans ranging in ages from 18 to 47 years and of at least 75% Polynesian heritage were given 10 mg of debrisoquine sulfate for the CYP2D6 study after overnight fasting. Of the 78 subjects who classified themselves as 100% Polynesian and the 22 who classified themselves as 75% Polynesian, the incidences of debrisoquine PMs were reported at $0\% \pm 3.6\%$ with metabolic ratios values from 0.01 to 9.94 [64].

In a proguanil study, 33 females and 26 males from the original group (ranging in ages from 18 to 44) were given 200 mg of proguanil hydrochloride, one week after the debrisoquine study and collected for 8 h and tested using the same protocol above; only the antimode metabolic ratio of proguanil:cycloguanil above 10 ($\log_{10} = 1$) indicated PMs. Of this group, eight subjects (five of 100% Polynesian decent, two of 25% Chinese descent and one of 25% Caucasian decent) were classified as proguanil PMs or 13.6% of the subjects with a 95% confidence interval of $5.9 \pm 24.6\%$ with metabolic ratios from 11.1 to 34.4. Compared to EMs, PMs excreted approximate 50% less compared to the amount given orally. The results of these studies showed that Polynesian subjects have a closer resemblance of CYP2D6 and CYP2D16 polymorphisms to that of Southeast Asian populations than to Caucasian populations, which may contribute to differences in kava metabolism and toxicity in Polynesian kava users [64].

The effect of mixed and/or Polynesian ancestry on the rate of CYP polymorphisms within a population has not been studied. Native Hawaiians, for example, have intermixed with many other populations such as Asians and Caucasians, and the prevalence of genes responsible for UM, EM and PM phenotypes is unknown. Diminished CYP2D6 functionality in Mexican American populations may provide a useful analogy that we can use to illustrate the potential for similar polymorphisms in Pacific populations. Mexican Americans (MA) have a diverse American Indian, Spanish, African, Caucasian genetic background and 2.6% of the population was classified as PMs [65]. Within the Native Hawaiian community, the results of an influx of Caucasian and other genes since the late 1700s may have affected the CYP2D6 polymorphisms of this population originating from those of Polynesian decent.

6.2. Drug interactions

Kavalactones inhibit several P450 enzymes, and as a result they could interact with drugs and herbal supplements. In addition to pharmacokinetic interactions, kava may have the potential to cause pharmacodynamic interactions as some of the lactones have exhibit the ability to obstruct gamma-aminobutyric acid (GABA) receptors and both sodium and calcium ion channels [66]. Several drugs

and drug categories have been reviewed for their potential interactivity with kava:

Kava is known for its calming and sedating effects and as such concerns arose to its potential to interact with central nervous system depressants such as alcohol, barbiturates and benzodiazepines [57,66]. Interactions with kava and alcohol have been reported in both mice and human subjects. In humans, the effect of kava (1 g/kg powder in 500 mL water) and alcohol (0.75 g/kg) both alone and together was tested on cognitive performance of 10 subjects. When both kava and alcohol were co-consumed, impairment was noticeably increased [57,66]. Interactions with Levodopa, aspirin and warfarin have also been proposed [57].

Interactions with kava and other herbal products are of potential concern. Several of the cases reviewed for kava toxicity have also indicated that St. John's Wort was co-ingested including one of the CYP2D6 deficient patients and a case involving an approximately 68-year-old woman presenting with cholestatic hepatitis [8]. St. John's Wort has shown inhibition on CYP enzymes, specifically intestinal CYP3A4. Moreover, St. John's Wort extracts in the U.S. must contain at least 3.0% hyperforin, and this compound interferes with the uptake of serotonin, norepinephrine and most importantly, dopamine [22,66]. Consequently, St. John's Wort is a potential inhibitor of any drug that affects these important brain neurotransmitters and since kava has also demonstrated inhibition of dopamine, they may well interact.

Even dietary foods and drinks can have interactions with medications or other supplements. For example, IC_{50} values for kavalactones at CYP1A2, 2C9, 2C19, 2D6, and 3A4 resemble those of the grapefruit components bergamottin, 6,7-dihydroxybergamottin and naringenin [60,57], and thus there is the potential for interactions of kava even with everyday ingestants.

6.3. Enantiomers of kava components and toxicity

Many biological structures and processes differentiate between chiral compounds. Enzymes, receptors and transporters have enantiomeric specificity. Chiral drugs are processed by biological systems imbued with this enantiomeric propensity and the pharmacokinetics and toxicity of enantiomers differ [67]. Kawain, dihydrokawain, methysticin and dihydromethysticin are chiral compounds [8]. The kava-metabolizing CYP2C19 shows enantiomer (metabolizing S faster than R) preference for mephenytoin, an anticonvulsant [62]. Some commercially available kava treatments add a racemic synthetic kawain, which is thought to increase activity but may instead increase toxicity [27]. Despite concerns that racemic lactones like kawain may be responsible for the toxicity of commercially prepared organic extracts, studies addressing this issue seem to be non-existent (to date). One study examined pharmacokinetics of naturally occurring (+)-

kawain enantiomer as well as the inhibition of P450 enzymes by naturally occurring kavalactones, but did not look at the effects of the other enantiomer [59].

Stereospecificity of enzymes in both the activation and elimination of chiral pharmaceutical drugs is critical in providing safe effective medications. In the absence of explicit kava studies on chirality, we can look to other traditional medicines for analogous studies: Wang and Zeng (2010) reviewed several studies that focused on Traditional Chinese Medicines (TCM) that contain at least one chiral center, which may also give a glimpse into the pharmacokinetics of the chiral components found in kava [67]. The first group, the citrus flavonoids (flavonones), contains a chiral carbon within a ring structure, which can undergo non-enzymatic conversion from one enantiomer to the other or racemization from optically active to inactive. Of these, three flavonones were tested in rats for their pharmacokinetics: hesperetin, naringenin and eridictyol. Hesperetin and naringenin enantiomers were metabolized differently [67,68].

The L-(–)-form of an alkaloid, tetrahydropalmatine (THP) from *Cordylis yanhusuo*, is much more active as a pain reliever than D-(+)-THP [67]. Hong *et al.* compared the effects of pharmaceutical grade rac-THP on rats with naturally occurring THP in plant extracts. Stereoselectivity was three times higher for the (–) than (+) THP; interestingly, rats exposed to mixed plant extracts containing THP had significantly higher values for (+)-THP suggesting that stereoselectivity is decreased as the chemical complexity increases [69]. This study underlines that the chemical complexity of herbal remedies has a different effect on pharmacokinetics than the purified known active ingredient. Likewise, the complexity of kava has often been argued, by traditional practitioners, to increase the efficacy and safety of this herbal supplement, and this is the first study to establish a potential mechanism for this hypothesis.

6.4. Kava strains and variability in manufacturing/preparation

Other potential causes, as suggested by traditional practitioners, of kava toxicity in non-traditional preparations are: (1) the use of leaves, stems and other plant parts in manufacturing caplets and tinctures instead of root material, and (2) the use of inappropriate cultivars in a manner uninformed by cultural experience and practice. Variances in the chemical composition between the roots, rhizomes and basal stems of the kava plant have been of concern. The alkaloid pipermethystine, a cytotoxin, has been isolated from aerial parts of the plant [27]. Six different potential products from the plant: roots, stems, basal stems, peelings and chips from the rhizome and residues, each traditionally having a specific definition and designated medicinal uses. It is important that nutraceutical manufacturers and novice kava users understand these differences. Pacific traditional drinkers of kava use only the peeled root, carefully washed and ground. In contrast, commercial kava products are often made from peelings and chips of the dried rhizome contaminated with basal stems in the interests of economy. A code for standardization has been explicitly proposed by Teschke *et al.* [70].

Traditional practitioners also have a sense of the most appropriate cultivars for specific uses. There are 200 known kava cultivars in four classifications: Noble, medicinal, Tu Dei (or Two Days) and Wichmannii cultivars. Noble cultivars are

considered by Pacific practitioners as the safest as no incidences of liver toxicity has been linked to their traditional social use. Tu Dei cultivars are known for their extended psychotropic effects lasting “two days,” hence their name; these cultivars have been associated with nausea, which is believed to be caused by a high concentration of dihydromethysticin. Finally, *Piper wichmannii*, the wild species from which the domesticated *P. methysticum* is derived, is not used for daily consumption due to its long lasting physical effects and low degree of beneficial effects. Supplement manufacturers and their regulators may not take into account the various cultivar classifications and their different effects. World Health Organization findings alluded to this as a potential cause of liver toxicity and the Secretariat of the Pacific Community expressed the importance of selecting the proper kava cultivar for export and establishing a set of standards to ensure the best possible kava to be used for nutraceuticals [47]. Notably, it may be that kava in Hawai'i is free of toxic effects because it represents the end-point of the cultivar selection process across the whole Pacific, so there are no Wichmannii or two day kavas among the Hawaiian varieties [48,71,72]. Thirteen contemporary cultivars found in Hawai'i are unique to Hawai'i, reflecting a process of selection and strain development that Hawaiian farmers engaged in after bringing cultivars from the Marquesas and through somatic mutation developed/selected the cultivars we have today on the basis of stem color and effect (Fig. 2) [48,71,72].

7. Benefits of kava consumption

Kava has been used in Europe since the 1880s to relieve stress and anxiety and British herbal practitioners have been using it to treat urinary cystitis, rheumatism, urethritis and urinary tract infections since the early 1900s [5,73–75]. Kava has a history of use as a nervine treating dizziness, melancholy and neuralgia [74]. More recently, there has been an increased use of kava to treat disorders such as anxiety, nervous tension, restlessness, insomnia and even mild depression and symptoms of menopause [76–92]. Trials of kava have demonstrated it to be superior in treating anxiety compared to placebos and has even been effective where other medications have not [93]. Additionally, kava does not appear to be addictive like alcohol and many prescription drugs; in addition, there is no association with violent or antisocial behavior and kava use [75,94].

7.1. Benefits suggested by the traditional Pacific pharmacopeia

Kava, at first glance, has a surprisingly broad indigenous pharmacology. Medicinally, kava has been used for a wide range of both CNS-centered and peripheral effects. The CNS-centered effects of kava are the most highlighted both in traditional practice, recreational use and contemporary nutraceutical marketing campaigns. These are the sedative and calming effects, which, in the world of nutraceuticals are promoted as treatments for stress, anxiety, and depression, often portrayed as “natural” analogs of anxiolytic and antidepressant pharmaceuticals. While the CNS-centered effects are the most widely cited and discussed, much of the broad list of traditional medicinal uses is not related to the CNS-centered effects. Peripherally, kava is indicated in traditional Pacific medicine for urogenital conditions (gonorrhea infections, chronic cystitis, difficulty urinating), reproductive and women's health

(for menstrual problems and dysmenorrhea, to facilitate delivery, to stimulate milk production, as an abortifacient and contraceptive), gastrointestinal upsets, respiratory ailments (asthma, coughs, and tuberculosis), skin diseases and topical wounds, and as an analgesic, with significant subtlety and nuance attending the precise strain, plant component (leaf, stem, root, etc.) and preparative method to be used [1–4,75]. These data suggest active components in kava that extend beyond the GABA-ergic, CNS-active, kavalactones, and that may be sufficiently varying with strain, component and preparative method to underlie the complexity that is present in the traditional pharmacopeia.

7.2. Potential chemopreventive actions of Kava

In spite of the evidence that suggests that kava consumption is linked to hepatotoxicity and tumorigenesis, other studies show kava as a potential chemopreventive. Dosing of a small number of mice with 10 mg/g of kava for 30 weeks showed a reduction in chemically-induced lung adenomas by over 50% [95,96]. These studies saw no liver toxicity due to kava exposure. The flavokawains A, B and C (FKA, FKB, FKC) were tested as possible kava components responsible for the tumor suppression. Flavokawains are chalcones, several of which have exhibited activity against a range of different types of cancers. Of the three, only FKB demonstrated any reduction in lung adenomas (34%) [96]. For example, Wattenberg *et al.* (1994) showed that 2-hydroxychalcone administered at a dose of 5 mg/g reduced lung tumor multiplicity by approximately 30 to 40 percent. Moreover, Zi *et al.* (2005) noted that flavokawain A suppressed tumor growth in bladder cells [52].

Several potential mechanisms of anti-carcinogenic activity were explored in the above studies. First, Proliferating Cell Nuclear Antigen (PCNA) levels were assessed as PCNA overexpression associates with transformation. Substantial increases in PCNA were observed in kava-exposed animals compared to controls [95]. Moreover, data on PCNA and Ki67 expression showed that anti-carcinogenic activity diminishes over time without continued kava treatment [96]. Caspase 3 upregulation and increased cleavage of poly ADP-ribose polymerase (PARP) were also noted in lung tumors from kava-treated mice, suggesting higher levels of apoptosis.

Another possible mechanism for the chemopreventive effect of kava is reduction in induced DNA damage. After normalization of DNA adduct abundance for the time-controlled group experiment, all six NNK-exposed, kava-fed groups exhibited reduction in all four DNA adducts with 7-pobG, O^2 -pobdT, and O^6 -prodG showing reductions between 30 and 40%; whereas, O^6 -mG demonstrated a 70 to 80% reduction. More importantly, the relative abundances of these four DNA adducts showed no differences at different time points after NNK exposure, leading to the idea that kava treatment inhibits DNA damage [97].

8. Conclusions and open questions

A comprehensive understanding of kava chemistry is of importance in assessing the future of kava exposure in both Pacific and global populations. This generates two key considerations. First, the type of extraction to be characterized has important implications. There is a tension between fidelity to the traditional aqueous extractions of primarily root samples, and the need to analyze organic extracts of



Fig. 2. Hawaiian cultivars of *Piper methysticum* illustrating stem and leaf characteristics used in traditional selection practices. Hawaiian names of cultivars are shown. Photography credits: Mr. Ed Johnston, (upper panels) Mr. Harry Brevoort (lower panels), Association for Hawaiian 'Awa, Hilo, Hawai'i.

aerial and root powders that are the major nutraceutical forms of commercialized `awa. There is good evidence that kava toxicity and efficacy are linked to extraction method. Since both traditional and commercial/organic extracts are public health issues, both need to be examined comprehensively.

8.1. Is kava toxic?

Despite the link to kava and liver toxicity demonstrated *in vivo* and *in vitro*, in the history of Western kava use, toxicity is still considered relatively rare. Only a fraction of the handful of cases reviewed for liver toxicity could be, with any certainty, linked to kava consumption and most of those involved the co-ingestion of other medications/supplements [8,57]. That means that the incident rate of liver toxicity due to kava is one in 60–125 million patients [12]. For Pacific traditional users, despite the much higher kavalactone exposure, `awa liver toxicity is either unheard of or unreported. Nevertheless, in rural areas of the Pacific, where hepatitis is endemic, liver disease that may be caused by kava consumption may be masked and reported as other causes [9]. It is difficult to say with any sense of accuracy since there is a shortage of epidemiology and public health data in Pacific populations who habitually use kava.

8.2. Beyond the kavalactones—is there an entourage effect in kava?

Kava plants are likely to contain a diverse secondary metabolome, with hundreds of compounds that can impact the physiological responses of human cells and tissues [27,28,98–100]. The focus of the `awa field upon the kavalactones is linked to the strong likelihood that these compounds' ligation of CNS GABA receptors is responsible for the relaxant and anxiolytic effects of the drink and its supplements [101,102]. However, the physiological (and possibly pathophysiological) effects of kava may be underestimated by a unilateral focus upon the kavalactones. The secondary metabolome of *Cannabis sativa* provides an analogy here. For decades the primary focus of the field, the marijuana growing community, and medicinal marijuana proponents has been on the major cannabinoid compounds Δ^9 -THC, cannabidiol and cannabinol. These are indeed the main CNS-active components but they and their derivatives comprise ~7 of the >400 known bioactive molecules in *C. sativa*. Indeed, until the so-called 'entourage' of terpenes, alkaloids, etc., was factored into cannabinoid pharmacology [103,104], our understanding of its mechanisms and breadth of effect was severely limited. Similarly, the `awa field may now benefit from examination of the *P. methysticum* 'entourage'.

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